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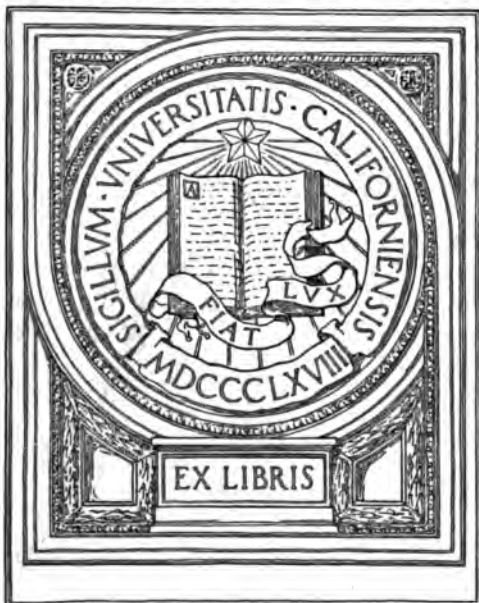


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# THE METABOLISM AFTER MEAT FEEDING OF DOGS IN WHICH PANCREATIC EXTERNAL SECRETION WAS ABSENT.

By FRANCIS G. BENEDICT AND JOSEPH H. PRATT.

*(From the Nutrition Laboratory of the Carnegie Institution of Washington,  
Boston, Mass., and the Laboratory of the Theory and Practice of  
Physic of Harvard University.)*<sup>1</sup>

(Received for publication, April 7, 1913.)

The well-known increase in the total metabolism of both animals and man resulting from the ingestion of various kinds of food has been the subject of much study, and while investigators are well in accord as to there being such an increase, they differ widely in their opinion as to the cause. At present there are two distinctly different theories. Unfortunately neither of these is sufficiently clarified to be sharply defined, and as experimental evidence accumulates there seems to be more or less of a tendency for the two theories to coalesce. In general it may be stated that the theory most actively championed by Zuntz and his co-workers assumes that the increase in metabolism is mainly due to the mechanical processes of digestion, including the work of peristalsis, segmentation, absorption, and glandular activity. Rubner, on the other hand, holds that this increase is due to the specific dynamic action of the foodstuffs, *i.e.*, a quota of heat production that appears as free heat and does not benefit the cells. It is thus seen that in one case the increase in metabolism is considered to be due chiefly to mechanical causes, while in the other it is mainly attributed to chemical processes.

Experimental evidence has accumulated to such an extent that we may be certain that an increased metabolic activity follows the ingestion of all of the three main nutrients—protein, fat, and carbohydrate. This is greatest in the case of protein; in fact, so

<sup>1</sup> This investigation was aided by a grant from the Proctor Fund for the Study of Chronic Diseases.

great is the increase in metabolism following the ingestion of protein that Zuntz himself is inclined to maintain that the work of digestion alone is not sufficient to account for this increased activity, and to accept the view that there is also a chemical action.<sup>2</sup> Rubner, on the other hand, is apparently unwilling to admit that the mechanical work of digestion plays any rôle.

A discussion of the general fundamental question of the specific dynamic action of foodstuffs and of the work of digestion is not appropriate to this paper, and is therefore deferred until the appearance of the results of a large amount of experimental work on man which is now being prepared for publication by Mr. T. M. Carpenter of the Nutrition Laboratory. An opportunity offered, however, for making a study of the metabolic changes following the ingestion of meat upon three dogs without external pancreatic secretion, whose power to digest and absorb protein was greatly diminished. While in recent years considerable evidence has accumulated as a result of surgical interference with the normal digestive tract, it has usually been obtained when the dogs were under the influence of curare or some narcotic, and the question has rightly been raised as to whether the results obtained under such conditions can be in any way normal.<sup>3</sup> Our three dogs, however, made an excellent recovery and continued in good health after the pancreatic secretion had been entirely excluded from the intestines; their absorption of protein and fat was then studied. Since absorption experiments with these dogs showed that a large amount of undigested meat was lost in the feces, it was evident that under these circumstances there would be a maximum work of digestion so far as peristalsis and segmentation were concerned, and a decreased absorption of material from the foodstuffs to be carried by the blood to the cells, there, according to Rubner's views, to produce, in part, free unutilized heat. It was believed, therefore, that a study of the metabolism of these animals to determine the effect of ingesting meat, if made with the proper technique, would throw light upon the general question of the cause of the increased metabolism following the ingestion of food.

<sup>2</sup> Zuntz and Loewy: *Lehrbuch der Physiologie des Menschen*, Leipzig, 1909, p. 678. See also, Loewy: *Oppenheimer's Handbuch der Biochemie*, Jena, 1911, iv, p. 271.

<sup>3</sup> Zuntz: *Oppenheimer's Handbuch der Biochemie*, Jena, 1911, iv, p. 855.

*Review of earlier work on the effect of the exclusion of the pancreatic secretion upon the absorption of food.*

Abelmann,<sup>4</sup> working under the direction of Minkowski, found that after the extirpation of the pancreas in the dog there was a great decrease in the absorption of nitrogen and fat; in fact, when meat was fed, there was no absorption of fat. After total extirpation of the pancreas, the percentage of nitrogen absorbed varied from 27.8 to 58 per cent. When pancreas was added to the diet, the absorption rose to 78 per cent. After partial extirpation of the pancreas, the absorption of nitrogen varied from 40 to 83 per cent. Unfortunately most of the experiments were of only one day's duration.

Rosenberg<sup>5</sup> made absorption studies on dogs in which he had separated the pancreas from the intestine. The absorption of nitrogen varied in experiments in which a meat diet was given from 64.1 to 83 per cent, while normally it was above 90 per cent. Lombroso<sup>6</sup> and also Zunz and Mayer<sup>7</sup> concluded from their experiments that all of the pancreatic juice could be excluded from the intestine without serious disturbance in the absorption of proteins and fats. These investigators stated that they tied "both" pancreatic ducts, but Hess<sup>8</sup> and his student Sinn<sup>9</sup> have shown that the pancreas in the dog has usually three ducts and sometimes four. In eight experiments on dogs, Hess<sup>10</sup> succeeded only twice in tying all the ducts at operation and these two animals were the only ones in his series in which the absorption of fats and proteins was definitely reduced. With one of these dogs, 54.68 per cent of nitrogen was absorbed, and with the other, 42 per cent.

Niemann,<sup>11</sup> working under the direction of Brugsch, also studied the absorption in dogs after tying the pancreatic ducts. Although the claim was made that no pancreatic juice entered the intestine, one dog was found to absorb 93.9 per cent of nitrogen and another dog 92.9 per cent. A meat diet was used in both experiments. Fleckseder,<sup>12</sup> investigating the same problem in Hans Meyer's laboratory, concluded that normal absorption can occur even when all the pancreatic juice is excluded from the intestine.

The observations published by Pratt, Lamson, and Marks<sup>13</sup> in 1909

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<sup>4</sup> Abelmann: Inaugural Dissertation, Dorpat, 1890.

<sup>5</sup> Rosenberg: *Pflüger's Archiv*, lxx, p. 371, 1898.

<sup>6</sup> Lombroso: *Pflüger's Archiv*, cxii, p. 531, 1906; *Arch. f. exp. Path. u. Pharm.*, lviii, p. 251, 1908.

<sup>7</sup> Zunz and Mayer: *Mém. couronnées et autres mém. p. p. l'Acad. Royale de Méd. de Belgique*, xviii, p. 68, 1904.

<sup>8</sup> Hess: *Pflüger's Archiv*, cxviii, p. 536, 1907.

<sup>9</sup> Sinn: Inaugural Dissertation, Marburg, 1907.

<sup>10</sup> Hess: *Medizin.-Naturwiss. Archiv*, i, p. 161, 1908.

<sup>11</sup> Niemann: *Zeitschr. f. exp. Path. u. Ther.*, v. p. 466, 1909.

<sup>12</sup> Fleckseder: *Arch. f. exp. Path. u. Pharm.*, lix, p. 407, 1908.

<sup>13</sup> Pratt, Lamson, and Marks: *Trans. of the Assoc. of Amer. Physicians*, xxiv, p. 266, 1909.

showed that the good absorption obtained by the earlier investigators was due to failure in excluding permanently the pancreatic secretion from the intestine. In experiments upon a series of four dogs, in which the pancreas had been separated from the duodenum, marked diminution in the absorption of fat and nitrogen was found. The absorption of nitrogen ranged from 22.2 to 61.7 per cent. In all of these animals, great atrophy and sclerosis of the pancreas occurred. The operations were made by Dr. Fred T. Murphy and in only one of five dogs did he fail to shut out the pancreatic juice from the intestine. In this animal, which was the first of the series, there was no decrease in absorption of either the protein or the fat. At the autopsy the pancreas was of normal size and consistence and a sinus was found connecting the interior of the duodenum with the main duct of the pancreas.

*Description of the operations on the dogs used in this research.*

*Zep.* One of the three dogs used in the present study, "Zep," was a female which, while a puppy and weighing 5.7 kgms., had had the pancreas separated from the duodenum. This was more than two and a half years prior to the date of our experiments in the respiration apparatus, the operation being performed by Dr. F. T. Murphy on November 19, 1908. Ether was used as the anaesthetic, and a linear incision was made in the middle line of the abdomen. The pancreas was about 10 cm. long. Two ducts were found and cut between double ligatures. All the connections between the pancreas and duodenum were severed except the main branches of the arteria and vena pancreatico-duodenalis. A portion of the omentum was then placed about the duodenum, so that the corpus pancreatis<sup>14</sup> and duodenum were not in contact at any point.

*Pat.* This dog was a medium-sized, healthy male, weighing 11 kgms. The operation was performed February 13, 1911. Prior to anaesthesia by ether, 3 cc. of 1 per cent solution of morphia sulphate were injected. On opening the abdominal cavity by the usual incision, the internal end of the spleen was found to be almost in the median line. The terminal portion of the processus uncinatus of the pancreas, which measured 5 by 3 cm., was separated from the gland by cutting between double ligatures of black silk. An incision was also made in the lower portion of the internal surface of the spleen; a large pocket was then made and the portion

<sup>14</sup> For nomenclature, see *Pföger's Archiv*, cxviii, p. 267, 1907.

of the processus uncinatus separated from the remainder of the gland was placed in this. The vascular stalk, which entered the extreme end of the processus uncinatus and which contained an artery and vein, both of goodly size, was preserved. There was some difficulty in introducing the pancreatic tissue into the pocket made in the spleen, and during this manipulation considerable blood was lost as there was constant oozing from the cut tissues of the spleen. Finally, all the pancreatic tissue, except about 1 cm. of the terminal portion, was within the splenic substance. The graft was secured in place by sutures and the omentum was drawn around the vascular stalk. The dog made a good recovery.

A second operation was performed by Dr. Murphy on March 7, 1911. An injection of morphine was given before etherization, the dog vomiting within ten minutes. Very little ether was required. A thin linear scar marked the site of the old operation wound. When the abdomen was opened, a large mass was found behind the coils of intestine with its anterior surface covered with mesentery. After careful dissection this tumor was found to be an abscess occupying the lower end of the spleen which had been drawn forward. This was opened and there escaped about 30 cc. of thin, pinkish, puriform material with a pleasant odor resembling that of heliotrope. There were many adhesions of mesentery to the internal surface of the spleen, and a nodule of pancreatic tissue about 2 cm. in size projected from the splenic tissue. There was no evidence of inflammation about the duodenum or pancreas. The mesenteric stalk which carried the blood vessels to the graft in the spleen was cut. The duodenum was stripped clean of pancreatic tissue and two ducts were found and cut between double ligatures; the pancreatico-duodenal artery and vein were spared. The body of the pancreas and the splenic process were then quickly extirpated, the weight of the pancreatic tissue being 19 grams.

*Flora.* The dog "Flora" was an old, fat, female dog, weighing 13.8 kgms. The operation was performed on March 29, 1912, by Dr. Beth Vincent. Under ether narcosis, the corpus pancreatis was carefully separated from the duodenum; the pancreatic ducts were cut between double ligatures, the main branches of the pancreatico-duodenal artery and vein being preserved. Strong ligatures were placed about the gland at the junction of the corpus with the processus lienalis above and at the boundary of the corpus

and the processus uncinatus below; the gland was then sectioned proximally to each of these ligatures and the corpus pancreatis removed. The excised tissue weighed 10 grams. Omentum was placed over the stumps of the processus lienalis and processus uncinatus.

*Methods for preparation and analysis of foods and feces.*

During the absorption experiments, the dogs were confined in metabolism cages. The diet proposed by Gies<sup>15</sup> was used in most of the experiments. This consisted of a mixture composed of 250 grams of chopped meat, 70 grams of cracker meal, 50 grams of bone ash, and 30 grams of lard. For demarcating the stools, carmine and charcoal were employed, but in the absorption experiment with "Flora" in which an exclusive meat diet was used, demarcation was obtained by feeding milk and bread, thus producing bright yellow feces which contrasted sharply with the dark meat stools. The method for determining the composition of the food was as follows: An aliquot part, as a fifth or a tenth, of the amount of each article of diet eaten daily by the dog was taken and placed in a large evaporating dish on the water bath. The composite mixture was analyzed as a whole. For the nitrogen determinations the Kjeldahl method was employed, a determination of the nitrogen in the meat used (finely chopped beef heart) being made for each experiment with the respiration apparatus.

*Absorption experiments with the dog "Zep."*

Nine absorption experiments were made with the dog "Zep." The details of the first experiment are as follows:

*First absorption experiment.* November 27-30, 1908; duration, 4 days; weight of dog November 27, 5.3 kgms.

Total food: 500 grams chopped beef heart; 100 grams bone ash; 140 grams cracker meal; 60 grams lard.

Weight of dried food, 425 grams; nitrogen in food, 4.59 per cent; amount of nitrogen, 19.5 grams.

Weight of dried feces, 265.5 grams; nitrogen in feces, 4.99 per cent; amount of nitrogen, 13.25 grams; nitrogen absorbed, 32.1 per cent; nitrogen lost in feces, 67.9 per cent.

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<sup>15</sup> Gies: *Amer. Journ. of Physiol.*, x, p. 22, 1904.

A summary of the results obtained in all of the absorption experiments with Zep is given in table 1. The absorption of nitrogen varied from 32.06 per cent to 60.20 per cent unless pancreas or pancreatic preparations were added to the diet.

The details of the absorption experiments with the dogs "Pat" and "Flora" are as follows:

*Absorption experiment with the dog "Pat."*

Date, March 20-22, 1911; duration, 3 days; weight of dog March 20, 8.125 kgms.

Total food: 750 grams chopped beef heart; 210 grams cracker meal; 120 grams bone ash; 60 grams lard

Weight of dried food, 438 grams; nitrogen in food, 4.73 per cent; amount of nitrogen, 20.78 grams.

Weight of dried feces, 342 grams; nitrogen in feces, 4.41 per cent; amount of nitrogen in feces, 15.08 grams; nitrogen absorbed, 27.92 per cent; nitrogen lost in feces, 72.08 per cent.

*Absorption experiment with the dog "Flora."*

Date, November 12-15, 1912; duration, 4 days; weight of dog November 12, 6.6 kgms.

Total food: 3000 grams chopped beef heart.

Weight of dried food, 767 grams; nitrogen in food, 10.87 per cent; amount of nitrogen, 83.31 grams.

Weight of dried feces, 395.3 grams; nitrogen in feces, 8.90 per cent; amount of nitrogen, 35.18 grams; nitrogen absorbed, 57.77 per cent; nitrogen lost in feces, 42.23 per cent.

*Measurements of the metabolism.*

Unquestionably the ideal measurements of the metabolism of the living organism include the determinations of the carbon-dioxide output, the oxygen intake, the heat production, and the usual analyses of food, feces, and urine. Such complete experiments are extremely expensive and time-consuming, so that they are justifiable only after preliminary experiments in which the factors affecting metabolism have been so carefully studied and controlled as to insure that the elaborate records for the oxygen determinations and the heat measurements will give results not vitiated by extraneous, uncontrolled factors. To be of any value, the determinations of the oxygen consumption must be accurate

TABLE 1.

*Summary of results obtained in absorption experiments with the dog "Zep."\**

EXPERIMENT NO.	DATE	DURATION	BODY WEIGHT	NITROGEN IN FOOD	NITROGEN IN FECES	TOTAL NITROGEN LOST IN FECES	DIET
		days	kilos	grams	grams	per cent	
1	1908 Nov. 27-30	4	5.3	19.5	13.3	67.9	500 gms. chopped beef heart 100 gms. bone ash 140 gms. cracker meal 60 gms. lard
2	1909 Feb. 2-5	4	5.5	78.9	31.4	39.8	1706 gms. chopped beef heart 341 gms. bone ash 478 gms. cracker meal 80 gms. lard
3	Apr. 4-7	4	6.25	44.1	25.4	57.6	1000 gms. chopped beef heart 200 gms. bone ash 280 gms. cracker meal 80 gms. lard
4	Apr. 12-15	4	6.25	42.3	18.6	44.0	1000 gms. chopped beef heart 200 gms. bone ash 280 gms. cracker meal 1.8 gms. of "holadin," a pancreatic preparation, given daily
5	Oct. 12-15	4	6.45	193.3	109.8	57.1	4000 gms. chopped beef heart 1050 gms. bone ash 100 gms. crushed bone 1200 gms. cracker meal 600 gms. lard
6	Dec. 1-5	5	6.3	21.9	10.0	45.5	3800 cc. milk
7	Dec. 7-12	6	6.1	36.3	16.4	45.3	6260 cc. milk
8	1910 Feb. 8-11	4	5.1	45.5	7.9	17.2	1000 gms. chopped beef heart 280 gms. cracker meal 80 gms. lard 200 gms. bone ash 200 gms. fresh sheep pancreas
9	1911 May 24-26	3	7.3	84.8	37.2	43.9	3000 gms. chopped beef heart.

\* Pancreas separated from duodenum and all pancreatic secretion excluded from intestine.



to within 5 per cent, at least, of the true amount. The same may be said regarding the heat measurements. Obviously, the nearer the results approach to accuracy, the greater their value. Fortunately the carbon-dioxide excretion may be determined with great exactness, so that when the dietetic conditions are constant, as was the case in this research, the errors incidental to the computation of the energy output from the carbon-dioxide production are reduced to a minimum, especially with normal animals.

*Respiration apparatus.* The carbon dioxide produced by the dogs used in these experiments was determined by means of a slightly modified form of the Benedict and Homans apparatus which has previously been described.<sup>16</sup> In this apparatus the animal remains in a closed chamber—in this instance, with a capacity of 1000 liters<sup>17</sup>—from which the air is continually withdrawn by means of a rotary blower. The ventilating current is first passed through sulphuric acid to remove the water vapor, then through soda lime to remove the carbon dioxide, and again through sulphuric acid to remove the small amount of moisture added to the dry air as it passed through the moist soda lime; it is then returned to the chamber. By this process the total amount of carbon dioxide produced is absorbed. As the oxygen of the air is consumed, the deficiency is supplied by admitting a fresh supply from a steel cylinder of compressed oxygen. The chamber itself is of galvanized iron, with a top which rests in a water seal. A rubber diaphragm made from a bathing cap is attached to a pipe at one side of the chamber, thus providing for slight expansions and contractions of the air in the system, and also controlling the admission of oxygen.

In the experiments in this study, the dogs were kept inside the chamber for twenty-four to thirty-six hours, the carbon dioxide being collected and weighed for each 4-hour period as it was possible to change from one set of air purifiers to another every 4 hours. The measurement of the oxygen consumption was attempted in only one series of experiments, later described.

<sup>16</sup> Benedict and Homans: *Journ. of Med. Res.*, xxv. p. 409, 1912.

<sup>17</sup> In certain experiments with a control dog "Clara" and in all the experiments with the dog "Flora" a smaller chamber was used, having a capacity of only 280 liters.

*Records of muscular activity.* As a result of experience with this apparatus we believe that every properly conducted metabolism experiment should consist of two inseparable parts, first, a record of chemical data for the carbon dioxide produced and, when feasible, for the oxygen consumption and heat production; and second and of equal value, a graphic record of the muscular activity or the degree of quietness of the subject. In no research thus far made in this laboratory have these graphic records of the degree of muscular activity or rest been of such vital importance. The method of obtaining these records, which is very simple, has already been described by Benedict and Homans.<sup>18</sup> One end of the cage in which the dog is confined inside the chamber rests on a knife edge bearing, the other end being supported by a strong spiral spring. The weight of the animal is thus equally divided between the knife edge and the spring. Beside the spring supporting the cage is placed a pneumograph supplied by the Harvard Apparatus Company, consisting of a rubber tube reinforced inside by a brass spiral spring. The elongations of the spiral spring are accompanied by similar elongations of the pneumograph, the confined air inside the rubber tubing expanding or contracting with each movement of the cage. This expansion or contraction of the air inside the pneumograph is transmitted to a rubber tube outside connected with a delicate Marey tambour which records with a pointer on a smoked drum. Every change in the center of gravity of the animal alters the tension of the spring, thus producing a corresponding movement of the pointer. With the small apparatus described by Benedict and Homans, it is possible so to adjust the sensitiveness of the apparatus as to record even the respirations of the dog, inasmuch as the slight change in the center of gravity of the animal during a respiration produces a change in the tension of the spring and a movement of the pointer. Such a degree of refinement was entirely unnecessary in these experiments and was not employed with the larger apparatus used for this study. The minor muscular movements, such as slightly moving the head, one of the legs, or even the tail, could, however, be easily identified, and during the whole progress of the experiment, a kymograph record was made of the movements of the cage.

<sup>18</sup> Benedict and Homans: *loc. cit.*

*Importance of kymograph records.* In studying the effect of any superimposed factor upon the fasting metabolism it is important to know whether an increase in metabolism, if noted, is due to extraneous muscular activity or to internal activity, but without a record of the external muscular activity, no adequate idea of the transformations in the body can be obtained. In very few of these experiments was there sufficient movement on the part of the dog to be visible to the observer and, in general, we might have stated that in practically all of the experiments the dog was resting quietly in the chamber. By means of the kymograph records, however, it was shown that there were noticeable variations in the muscular activity in the different periods—variations that certainly produced a change in the metabolism not due to the internal activity. The method of selecting periods for comparison was as follows:

The kymograph records were carefully examined by an assistant and from the excursions of the tambour, each period was classified as one of a number of degrees of activity varying from very quiet to moderately active. An independent estimate of the variations in muscular activity was subsequently made by the same person. This duplicate estimate was repeated by another assistant, so that four estimates were made which agreed remarkably well. All periods estimated as active or above were discarded, and only those periods which were considered as quiet were used for comparison. An exact estimate of the relative values for the experimental periods was of course difficult, but it is probable that a reasonably approximate estimation was secured and, that the periods selected for comparison are at least fairly representative of the conditions inside the chamber.

In this way and this way only can we be sure of suitable and proper bases for a comparison of the metabolism in the different periods. It would be obviously illogical to compare the amount of carbon dioxide per hour produced during fasting with muscular relaxation and quietness with the amount produced after feeding when there was considerable extraneous muscular activity, as otherwise the effect due to the ingestion of food would be greatly magnified. Conversely, if during the fasting period the dog was unusually restless, and after feeding was quiet, the increase due to the ingestion of food would be too small.

*Plan of investigation.* The investigation began the end of May, 1911, and continued almost uninterruptedly until the middle of July, 1911. Since the apparatus was in use nearly every day for experiments with the two animals with atrophied pancreas, it was impracticable to conduct simultaneous experiments with a control animal. Furthermore, it was believed that the researches of Rubner, and more recently of Lusk, were practically identical in the results obtained with normal dogs, so that we could properly use these values for comparison with our results. Subsequently it was considered advisable to conduct control experiments with a dog of essentially the same body-weight as that of the dogs previously used and with the same apparatus, obtaining particularly the records of the muscular activity, since neither Rubner nor Lusk had controlled their experiments with such records. These control experiments were carried out in the fall of 1911 with a normal dog, "Clara."

After the completion of the experimental period with the normal dog, it was seen that the computation of the energy output of these dogs from the carbon-dioxide output might be open to the objection that while the normal dog after being fed with meat might be burning pure protein, the dogs which had been operated upon might be burning both protein and fat; under these conditions the calorific equivalent of the carbon dioxide would be very different, so that the results would not be comparable. It became necessary, therefore, to measure the oxygen consumption in order to determine the character of the catabolism of the dogs after operation. Accordingly, in the fall of 1912, modifications were made in the Benedict and Homans apparatus by means of which accurate determinations of the oxygen consumption could be obtained in addition to the determinations of the carbon-dioxide production. The accuracy of such measurements was frequently checked by burning alcohol inside the chamber and determining the respiratory quotient, the results obtained in a considerable number of half-hour periods showing a respiratory quotient for alcohol of 0.66 or 0.67, the theoretical value being 0.666. We therefore felt justified in using the apparatus for measuring the oxygen consumption, and this was done during the fall of 1912 in a series of experiments with "Flora," another dog in which all pancreatic juice had been excluded from the intestine.

*General routine of experiments.* In the fasting experiments, which were taken as a base line, the dogs were brought to the laboratory in the morning approximately 20 to 24 hours after the last meal, this meal usually consisting of 300 grams of meat and 300 cc. of milk. After the dogs had been weighed, they were placed inside the cage in the respiration chamber, the cover of the chamber was put on, and the ventilating current of air started. The experiment was then continued for six or more 4-hour periods, the carbon-dioxide production being measured for each period. Unless otherwise stated, the temperature of the air in the chamber was between 25° and 27°C., the experience of Benedict and Homans having shown that 25°C. is usually the optimum temperature for dogs. The feeding experiments were carried out according to the same routine except that just prior to the experiment, the dog was fed meat varying in amount from 500 to 1000 grams.

*Calculation of the results.* The total carbon-dioxide excretion was measured in all cases but, instead of presenting the data on the basis of the carbon-dioxide excretion per kilogram of body-weight, we have taken the average weight of the dogs as 7 kgms. and computed the results on the basis of a 7-kgm. dog. Since all four dogs used in this research were of nearly the same weight, i.e., about 7 kgms., this method of computation was even more satisfactory than if there had been a great variation in weight. Furthermore, the discussion of the results is wholly upon a *comparative* rather than an absolute basis.

#### *Respiration experiments with the dog "Zep."*

*Experiments without food.* Eight fasting experiments were made with the dog "Zep," the results of which are given in table 2. It was necessary to reject at least three periods<sup>19</sup> on account of the gross muscular activity indicated by the kymograph records, and the results for these periods are not included in the average for the experiments. In all of the experiments, with the exception of the last two, the average carbon-dioxide production per 4 hours remained reasonably constant, ranging from 19 to 22.2 grams. The last two experiments, which are characterized by a continued

<sup>19</sup> In many periods the dog was so restless as not to warrant a measurement of the carbon dioxide.

decrease in body-weight, showed a somewhat less carbon-dioxide production per hour. When computing the results on the basis of a 7-kgm. dog, the variations are from 18.1 grams to 21.8 grams, the average for the first seven experiments being 20.4 grams of carbon dioxide per hour. The low values found during the two experiments in July may possibly be accounted for by the excessive heat at that time and by the growing weakness of the animal. Since, however, the number of fasting experiments was approximately that of the feeding experiments, and they covered chrono-

TABLE 2.

*Carbon-dioxide production in experiments without food with the dog "Zep."*

EXPERIMENT NO.	DATE	BODY-WEIGHT WITH- OUT FOOD	CARBON-DIOXIDE PRODUCTION IN 4-HOUR PERIODS						AVERAGE CAR- BON-DIOXIDE PRODUCTION IN 4 HOURS	
			First period	Second period	Third period	Fourth period	Fifth period	Sixth period	At actual body- weight	On basis of 7 kilos body- weight
	1911	kilos	grams	grams	grams	grams	grams	grams	grams	grams
3	May 29		22.1						22.1	21.5*
4	31	7.19		21.6	21.4				21.5	20.9
8	June 7	7.27	21.1	19.2					20.2	19.4
13	13	7.12	23.5	22.0	21.1				22.2	21.8
18	20	6.96	23.3	16.8	16.9				19.0	19.1
24	27	6.92	24.7	19.9	20.2				21.6	21.8
30	July 5	6.66			16.1	17.9	17.0	17.6	17.2	18.1
	Average								20.5	20.4
37	July 13-14	6.44	12.2	15.3	15.4	15.8	13.5		14.4	15.7

\* Calculated by means of weight assumed from May 31.

logically about the same time, we believe that the average results of all the fasting experiments may properly be compared with the average of the feeding experiments; hence the average fasting value of 20.4 grams for this dog is taken for a basal value.

One exception to this is made in the case of the fasting experiment of July 13-14. The low value then obtained, *i.e.*, 15.7 grams of carbon dioxide for 4 hours on the basis of a 7-kgm. dog has been used for comparison with a feeding experiment on the following day. For this reason the value for the experiment of

July 13-14 is not included in the average and the results are given separately in the table.

*Feeding experiments.* In the feeding experiments the animal was given at one meal the total amount of meat ingested just prior to entering the respiration chamber. The measurements of the carbon-dioxide production were made in periods of four hours each, and in some instances when excessive amounts of meat were

TABLE 3.

*Carbon-dioxide production in experiments with food with the dog "Zep."*  
(Calculated to basis of 7 kilos of body-weight.)

EXPERIMENT NO.	DATE	BODY-WEIGHT WITHOUT FOOD	MEAT EATEN	CARBON-DIOXIDE PRODUCTION IN 4-HOUR PERIODS					
				First period	Second period	Third period	Fourth period	Fifth period	Sixth period
	1911	kilos	grams	grams	grams	grams	grams	grams	grams
5	June 1	7.19	1000			35.1	31.5	29.6	29.2
7	5-6	7.27	1000	32.5	32.5	36.1	28.4	28.0	24.2*
11	10	7.34	500		31.4	21.5	20.7	24.5	
14	14	6.97	500	28.4	23.4	24.2	25.5	19.0†	19.0†
16	17	7.02	500	27.8		20.2	20.2	18.4	
19	21	6.63	500	26.9		24.3	21.2	18.2	
22	24	6.93	500	23.5	32.9	21.7	22.8	21.3	
26	29	6.66	500	29.0	28.9	27.0	18.9	16.7	21.3
28	July 1	6.66	750	28.5	35.5	30.9	27.7	23.5	24.5
32	7	6.58	500				22.4	17.7	17.4
34	10	6.49	750						21.7‡
38	14-15	§	750	27.5		26.5	24.8	20.9	

\* The production for the seventh period was 24.5 grams and for the eighth period 23.5 grams.

† Average production per 4 hours for a total of 8 hours. The results for two 4-hour periods have been combined because of a probably deficient absorber in the first of the two periods. The total carbon-dioxide measured was 37.8 grams.

‡ The production for the seventh period was 20.2 grams.

§ The weight used in calculating results to the 7-kilos basis was 6.44 kilos obtained on July 13.

given, the experiments were continued for thirty-two hours, or eight periods of four hours each. The data for the feeding experiments are given in table 3, which records the body-weight of the animal just prior to the ingestion of the food, the amount of meat eaten, and the carbon-dioxide production per 4 hours for the various periods. In tabulating these results, a relatively large number of periods were rejected owing to the fact that the kymograph records showed that the muscular activity was too great

to admit of any comparison with the results of the fasting period. It should be stated that this selection of periods by means of the kymograph records was made without reference to the results obtained in the measurement of the carbon-dioxide production and, indeed, the periods were rejected before a careful inspection of these data was made. Although the table is not meant for general comparison purposes, it can be seen from the results given that as a rule the larger the amount of meat fed, the larger the carbon-dioxide production.

TABLE 4.

*Increase in the carbon-dioxide production following the feeding of meat in experiments with the dog "Zep."*

(4-hour periods.)

EXPERIMENT NO.	DATE	MEAT EATEN	INCREASE* IN CARBON-DIOXIDE PRODUCTION							
			First period	Second period	Third period	Fourth period	Fifth period	Sixth period	Seventh period	Eighth period
	1911	grams	grams	grams	grams	grams	grams	grams	grams	grams
5	June 1	1000			14.7	11.1	9.2	8.8		
7	5-6	1000	12.1	12.1	15.7	8.0	7.6	3.8	3.1	3.3
11	10	500		11.0	1.6	.3	4.1			
14	14	500	8.0	3.0	3.8	5.1	-2.8			
16	17	500	7.4		-2	-2	-2.0			
19	21	500	6.5		3.9	.8	-2.2			
22	24	500	3.1	12.5	1.3	2.4	.9			
26	29	500	8.6	8.5	6.6	-1.5	-3.7	.7		
28	July 1	750	8.1	15.1	10.5	7.3	3.1	4.1		
38	14-15†	750	11.8		10.8	9.1	5.2			

\* For fasting value for 4 hours, i.e., 20.4 grams (except July 14-15), see table 2.

† For July 14-15 the fasting value used is 15.7 grams obtained on July 13-14 and calculated to basis of 4 hours and 7 kilos of weight.

*Increase in carbon-dioxide production following the feeding of meat.* With the dog "Zep" the amount of meat fed varied from 500 to 1000 grams; the increments in the amount of carbon dioxide produced as a result of this feeding of meat have been computed and are given in table 4.

The average carbon dioxide per 4 hours in the fasting experiments being 20.4 grams, it will be seen from the results given in table 4 that in certain periods over 50 per cent more carbon dioxide was given off by the dog after eating meat than when fasting;



in fact, in the third period of the experiment on June 5-6, after the ingestion of 1000 grams of meat, the increase in the carbon-dioxide production was nearly 75 per cent, the increment being notable in all of the 4-hour periods in this experiment. The two experiments in which this large amount of meat was given showed that 1000 grams of meat were altogether too much to be ingested at one time, and thereafter the amount given was only 500 to 750 grams. With this smaller amount of meat, the increments were considerable during the first three or four 4-hour periods, but in no case when 500 grams of meat were ingested was the increase toward the end of the experiment of any size, usually reaching its limit at the fourth period. In the experiment of July 1, when 750 grams of meat were given, the increase in the carbon-dioxide production continued through all six periods, the rise and fall in the increased production being comparatively consistent. The same amount of meat was given on July 14-15, when the dog was obviously in a different condition physiologically; this, however, was not as successful as the first, inasmuch as the results of two periods were lost.

*Total 24-hour increment in the carbon-dioxide production following the feeding of meat.* While the increments for 4-hour periods are of interest in showing the time relations of the metabolism, the total effect of the meat feeding is best shown by noting the increment for the whole twenty-four hours. This has been computed for the experiments with the dog "Zep," and the results given in table 5. The increment shown is, in general, roughly proportional to the amount of meat ingested, although 750 grams may produce as great an increment as do 1000 grams of meat. The percentage of increment is of value for subsequent comparison with the results found in experiments with the normal dog "Clara."

*Physical condition during observation.* On May 31, 1911, about the time the experiments in the respiration apparatus were begun, the dog "Zep" weighed 7.19 kgms. When the feeding of the pancreas was stopped on April 15, the dog weighed 8.2 kgms. There was a gradual loss of weight from this time until the death of the animal and a lowering of the limit of assimilation for glucose, but diabetes did not develop. During the last two weeks of the experiments the dog appeared at times rather feeble and less playful. On July 18, after the completion of the respiration experiments, the dog weighed 6.19 kilograms. On August 18, the weight was 5.37 kgms. The observation was made at this time that she was thin but active and had

gained in strength during the previous month. In spite of the addition to the diet of fresh pancreas, which had been omitted during the respiration experiments, the dog slowly lost in weight and died on October 6, 1911. On the day preceding her death, she was out in the yard running about and was active and fairly strong, being able to jump into her cage which was elevated a foot or more from the floor. The last two weeks before her death, the stools were bulky, soft and fatty.

*Results of autopsy.* At the autopsy, no pancreatic tissue was found. The site of the corpus pancreatis was occupied by a small dense fibrous mass measuring about 2 cm. by 8 mm. by 2 mm., its outline merging into the surrounding connective tissues. The processus lienalis and processus uncinatus were entirely atrophied. A microscopic examination of the fibrous nodule showed scattered foci of epithelial cells in dense connective tissue but nothing that resembled pancreatic tissue.

TABLE 5.

*Increase in the carbon-dioxide production in twenty-four hours following the feeding of meat in experiment with the dog "Zep."*

EXPERIMENT NO.	DATE	MEAT EATEN	CARBON-DIOXIDE PRODUCTION IN 24 HOURS WITHOUT FOOD	INCREASE OVER FASTING	
				Apparent actual increase*	Proportion of increase
	1911	grams	grams	grams	per cent
5	June 1	1000	122†	69‡	57
7	5-6	1000	122†	59	48
11	10	500	122†	24§	20
14	14	500	122†	20	16
16	17	500	122†	16**	13
19	21	500	122†	19**	16
22	24	500	122†	19	16
26	29	500	122†	24	20
28	July 1	750	122†	48	39
38	14-15	750	94††	53††	56

\* The amounts here given have been obtained by combining increases shown in table 4, excluding all minus quantities and also positive quantities of 1 gram or less.

† Obtained by multiplying by 6 the average results for 4 hours as brought to the 7-kilos basis (see table 2).

‡ From the results in the corresponding periods of June 5-6, it is assumed that the increase in the first and second periods of June 1 would total about 25 grams.

§ From the average of the increases for the first period on other days when 500 grams of meat were eaten, an increase of 7 grams is assumed for the first period of June 10. Because of increase above 1.0 gram in the fifth period, 0.3 gram increase in the fourth period has also been included in the total increase.

\*\* From the average of the increases for the second period on other days when 500 grams of meat were eaten, an increase of 9 grams is assumed for the second period of June 17 and of June 21.

†† Obtained by multiplying by 6 the average result for 4 hours in the experiment of July 13-14 as calculated to the basis of 7 kilos of weight, i. e., 15.7 grams (see table 2).

‡‡ From the results in the first and third periods it is assumed that the increase for the second period would be 11 grams. The increase for the sixth period is assumed to be 5 grams.

*Respiration experiments with the dog "Pat."*

*Experiments without food.* Seven experiments without food were made with the dog "Pat," the results of which are given in table 6. The first six experiments were only twelve hours long and although the last experiment continued for twenty-four hours, it was necessary to reject the results of three periods on account of the muscular activity. In this table, also, the values are given for the average carbon-dioxide excretion during 4-hour periods, computed on the basis of 7 kgms. of body-weight. It will be seen that, on

TABLE 6.

*Carbon-dioxide production in experiments without food with the dog "Pat."*

EXPERIMENT NO.	DATE		BODY-WEIGHT	CARBON-DIOXIDE PRODUCTION IN 4-HOUR PERIODS						AVERAGE CARBON-DIOXIDE PRODUCTION IN 4 HOURS	
				First period	Second period	Third period	Fourth period	Fifth period	Sixth period	At actual body-weight	On basis of 7 kilos body-weight
	1911		kilos	grams	grams	grams	grams	grams	grams	grams	grams
6	June	2	7.27	21.7		21.8				21.8	21.0
9		8		25.9	23.6	20.8				23.4	23.1*
12		12	7.82		23.5	24.6				24.1	21.6
17		19	6.86	22.6	22.8	21.0				22.2	22.7
20		22	7.43	20.1	18.4	17.3				18.6	17.5
23		26	7.81	23.7	24.4					24.0	21.5
29	July	3	7.99				22.7	22.8	21.0	22.2	19.4
	Average									22.3	21.0

\* Calculated by means of weight (7.09 kilos) assumed from June 9.

this basis, the average carbon-dioxide production in a 4-hour period for the seven experiments was 21 grams, the results ranging from 17.5 grams to 23.1 grams. On the other hand, the weight of the dog fluctuated, the lowest weight being 6.86 kgms. on June 19, and the highest 7.99 kgms. on July 3. With such sudden changes in the body-weight, it is to be questioned whether a computation can justly be made on this basis, although we are using this method of computation in practically all experiments. Since in the discussion of the results, we are dealing with averages rather than with individual experiments, it may be assumed with this dog

also that the average carbon-dioxide excretion in a 4-hour period, computed on the basis of 7 kgms. of body-weight, may be taken as the base line for comparison with the results of feeding experiments with the same dog, and in subsequent comparisons the value of 21 grams will be so used.

*Feeding experiments.* Nine feeding experiments were made with the dog "Pat," each of twenty-four hours' duration, in which amounts of meat varying from 500 to 750 grams were eaten by the dog immediately before he was placed in the chamber. The results, calculated on a 7-kgm. basis, are given in table 7.

TABLE 7.

*Carbon-dioxide production in experiments with food with the dog "Pat."*

(Calculated to basis of 7 kilos of body weight.)

EXPERIMENT NO.	DATE	BODY-WEIGHT WITHOUT FOOD	MEAT EATEN	CARBON-DIOXIDE PRODUCTION IN 4-HOUR PERIODS					
				First period	Second period	Third period	Fourth period	Fifth period	Sixth period
	1911	kilos	grams	grams	grams	grams	grams	grams	grams
10	June 9	7.09	500	29.4	29.2	26.1	27.8	21.9	20.4
15	16	7.22	500	26.8		26.7	29.9	23.7	23.0
21	22	7.43	500	25.2	29.3	26.1	25.1	19.7	18.4
27	30	7.85	750		26.2	30.1	26.9	24.2	23.5
33	8-9	7.91	500			21.9	20.2	17.5	17.3
36	12-13	*	750				23.5	21.1	17.0

\* The weight used in calculating results to the 7-kilos basis is 7.85 kilos taken at 12 noon, July 11.

During certain days in July, the temperature of the air in the chamber rose considerably above 25°C., owing to the extreme heat at this time. Unfortunately with this dog a large number of periods, in fact, three entire experiments, had to be rejected owing to extraneous muscular activity, and it will be seen that in general all of the experiments after the first three were complicated by this excessive activity. It would appear from the results that the high temperatures incidental to the season were not well borne by the dog. While occasionally individual periods could be selected for computation of the results, on the whole the experimental evidence obtained after June 22 which can properly be used for discussion is very meagre. It should be emphasized here that during this whole time the dog was not so restless nor moved

about sufficiently to cause particular comment; the kymograph records, however, showed a continual disturbance and restlessness which in respiration experiments carried out with no records of the muscular activity would have been considered as entirely normal, and the results would have been used for drawing deductions. As previously stated, in no research thus far carried out in this laboratory have the kymograph records been of such value as in this study.

*Total 24-hour increment in the carbon-dioxide production following the feeding of meat.* The total increment in the carbon-dioxide production for twenty-four hours is of particular value for com-

TABLE 8.

*Increase in the carbon-dioxide production in twenty-four hours following the feeding of meat in experiments with the dog "Pat."*

EXPERIMENT NO.	DATE	MEAT EATEN	CARBON-DIOXIDE PRODUCTION IN 24 HOURS WITHOUT FOOD	INCREASE OVER FASTING	
				Apparent actual increase*	Proportion of increase
	1911	grams	grams	grams	per cent
10	June 9	500	126†	29	23
15	16	500	126†	31	25
21	22	500	126†	22	17
27	30	750	126†	31	25

\* The amounts here given have been obtained by the method used for "Zep" in table 5, i.e., by combining increases found for the 4-hour periods, excluding all minus quantities and also positive quantities of 1 gram or less.

† Obtained by multiplying by 6 the average result for 4 hours as brought to the 7-kilos basis; see table 6.

parison with the increment found with the normal dog; values for experiments after feeding 500 grams and 750 grams of meat were calculated on the same basis as those for "Zep" and are given in table 8. The noticeable feature of this table is the fact that the increase in the carbon-dioxide production after feeding 750 grams of meat is not appreciably greater than that found after feeding 500 grams.

*Physical condition during observation.* No sugar was found in the urine of this dog after the operation on February 13, 1911, but there was a progressive loss in weight after the extirpation of the pancreas on March 7, 1911, until April 4, 1911, when the weight had dropped as low as 7.15 kgms. From that time until the middle of August, 1911, there were only relatively

slight fluctuations, the range in weight being from 6.9 kgms. to 7.6 kgms. On March 25, 1911, the dog was found lying in the cage, being unable to stand on account of weakness. The feeding of pancreas was then begun and continued daily except on those days when the experiments were made in the respiration apparatus. After fresh pancreas was added to the diet, the dog gained slowly in strength; the stools continued soft and voluminous. On August 17, 1911, a record was made that the dog seemed fairly strong. During the following week the limit of tolerance for glucose was found to be about 15 grams. During the first week of September the dog vomited much of his food and grew quite weak. On September 10, 1911, he was found lying in the cage unable to rise. The animal was then killed with chloroform; after death, the dog weighed only 5.71 kgms.

*Results of autopsy.* An abscess was found in the lower part of the spleen measuring about 4.5 cm. by 3.5 cm. In the thick dense wall of this abscess was a wedge-shaped mass of fibrous tissue extending into the spleen for a distance of about 2 cm. There were no pancreatic remains at the site of the pancreas, but a microscopical examination showed a small amount of pancreatic tissue in the center of the fibrous nodule which extended from the abscess wall into the spleen. The pancreatic transplant, measuring about 3 mm. by 1 cm., consisted of scattered groups of acini separated and surrounded by connective tissue. No islands of Langerhans were found.<sup>20</sup>

#### *Control experiments with the normal dog "Clara."*

It should be stated at the outset that if the experiments with the normal dog "Clara" had been made primarily for the purpose of studying the physiological effects of the ingestion of protein, a somewhat different plan would have been followed, but it seemed desirable to make these control experiments with so far as possible the same technique and routine as in the experiments with "Zep" and "Pat." The experiments should be considered, therefore, only as control experiments and not as a definite study of the metabolism of a normal dog as influenced by the ingestion of meat.

In the fall of 1911, a young, healthy, female dog, "Clara," was secured, weighing approximately 7 kgms. This dog proved to be a very satisfactory subject for experiments of this kind, as she became quickly accustomed to the technique and for the most part was very quiet, so that fewer periods were rejected on account of muscular activity than with any of the other dogs. The almost entire absence of muscular activity in the large apparatus was

<sup>20</sup> The histological details of the case are described by Pratt and Murphy: *Journ. of Exp. Med.*, xvii, p 252, 1913.

controlled by subsequent experiments in the small apparatus; in practically all experiments without food the dog had a minimum activity, the values found in both apparatus agreeing remarkably well.

*Experiments without food.* The results obtained in experiments without food and with the apparatus used for the experiments with "Zep" and "Pat" are given in table 9, and show that the average carbon-dioxide production for a 4-hour period on a 7-kgm. basis was 20.4 grams.

*Feeding experiments.* All of the experiments with food were made with the large chamber. Either 500 or 750 grams of meat were given the dog, since it was found impracticable to use as

TABLE 9.

*Carbon-dioxide production in experiments without food with the dog "Clara."*

EXPERIMENT NO.	DATE	BODY-WEIGHT	CARBON-DIOXIDE PRODUCTION IN 4-HOUR PERIODS				AVERAGE CARBON-DIOXIDE PRODUCTION IN 4 HOURS	
			First period	Second period	Third period	Fourth period	At actual body-weight	On basis of 7 kilos body-weight
	1911	kilos	grams	grams	grams	grams	grams	grams
41	Oct. 21	6.86	20.4				20.4	20.8
42	23	7.24		22.3	19.5	20.8	20.9	20.2
44	26	7.11	21.5	19.2			20.4	20.1
	Average						20.6	20.4

large an amount as 1000 grams, this amount being used only in the experiments with the dog "Zep." The results of the feeding experiments are given in table 10. While the data are not presented for the purpose of comparing the metabolism following the ingestion of varying amounts of meat, it will be seen that the results obtained with 500 grams of meat are approximately constant, and that on the one satisfactory day when 750 grams of meat were fed, namely, November 13-14, there was a much larger output of carbon dioxide than on the days when 500 grams of meat were fed.

*Total 24-hour increment in the carbon-dioxide production following the feeding of meat.* The total 24-hour increases found with the dog "Clara" are given in table 11, from which it is seen that the increase after feeding 750 grams of meat was greater than after

500 grams, the three experiments in which 500 grams were ingested agreeing very well.

TABLE 10.

*Carbon-dioxide production in experiments with food with the dog "Clara."*  
(Calculated to basis of 7 kilos of body-weight.)

EXPERIMENT NO.	DATE	BODY-WEIGHT WITHOUT FOOD	MEAT EATEN	CARBON-DIOXIDE PRODUCTION IN 4 HOURS					
				First period	Second period	Third period	Fourth period	Fifth period	Sixth period
	1911	kilos	grams	grams	grams	grams	grams	grams	grams
45	Oct. 26-27	7.11	500	29.8	28.6	28.8	26.8	25.7	21.3
47	30-31	7.07	500	28.7	27.7	28.5	26.2	23.0	19.8
48	Nov. 2-3	7.06	500	32.1	28.5	27.5	27.3	19.6	20.0
54	13-14	6.95	750	31.0	37.5	33.5*	33.4*	24.9†	24.8†

\* These results were calculated from the combined amounts for the third and fourth periods because of a deficient absorber in the third period.

† These amounts were calculated from the combined results for the fifth and sixth period because of a deficient absorber in the fifth period. The production for the seventh period was 26.3 grams when calculated to the basis of 4 hours and 7 kilos of weight.

TABLE 11.

*Increase in the carbon-dioxide production in twenty-four hours following the feeding of meat in experiments with the dog "Clara."*

EXPERIMENT NO.	DATE	MEAT EATEN	CARBON-DIOXIDE PRODUCTION IN 24 HOURS WITHOUT FOOD	INCREASE OVER FASTING	
				Apparent increase*	Proportion of increase
	1911	grams	grams	grams	per cent
45	Oct. 26-27	500	122†	38	31
47	30-31	500	122†	32	26
48	Nov. 2-3	500	122†	34	28
54	13-14	750	115‡	71	62

\* The amounts here given have been obtained by the usual method, i.e., by combining the increases for the 4-hour periods excluding all minus quantities and also positive quantities of 1 gram or less.

† Obtained by multiplying by 6 the average result for 4 hours as brought to the 7-kilos basis; see table 9.

‡ Obtained by multiplying by 6 the average result for 4 hours in the experiments of November 11 and November 13 with the small apparatus, i.e., 19.1 grams as brought to basis of 7 kilos of body-weight.



*Respiration experiments with the dog "Flora."*

The experiments with this dog were all carried out in the fall of 1912, immediately following the development of a method for determining the oxygen in the respiration apparatus, so that the amount of oxygen measured could be obtained as well as the carbon dioxide produced. It is unnecessary to give here the method of determining the oxygen consumption other than to say that it was based upon the fundamental principle carefully worked out for the large respiration chamber in this laboratory.<sup>21</sup> The small apparatus with a capacity of 280 liters was used in all of the experiments with this dog, the respiration chamber being surrounded with a water bath which was kept by a Reichert thermostat at a temperature of 29° to 30°C.; under these conditions, the animal lay perfectly quiet for an hour or more at a time, so that sharply defined periods were easily obtained. The dog was unusually tractable, very quiet, and an ideal subject in every way. No long experiments were made, as the primary object of the study was the determination of the character of the catabolism.<sup>22</sup>

*Experiments without food.* During the fall of 1912, a number of short-period experiments were made with this dog after it had fasted sixteen or more hours. The results of five of these experiments, each of which followed a fast of twenty-four hours, are given in table 12, showing an average carbon-dioxide production during 4 hours of 17.2 grams.

*Feeding experiments.* In the feeding experiments, instead of keeping the dog in the chamber continuously as was done with "Zep" and "Pat," the animal remained in the chamber only when she was being studied after the feeding. Satisfactory results were obtained on four different days with this dog, 750 grams of meat being fed in all instances, excepting on November 20, when only 700 grams were fed. The results for these experiments are given in table 13.

*Increase in carbon-dioxide production following the feeding of meat.* Assuming that the fasting base line for this dog on the basis

<sup>21</sup> Benedict and Carpenter: Carnegie Institution of Washington, Publication No. 123, 1910.

<sup>22</sup> We are indebted to Dr. Sergius Morgulis of the Nutrition Laboratory staff for valuable assistance in these experiments.

of 7 kgms. of body-weight was 17.2 grams of carbon dioxide per 4 hours, we can readily compute the increment due to the ingestion of meat. Thus, in table 13 we find three values for the first

TABLE 12.

*Carbon-dioxide production in experiments without food with the dog "Flora."*

DATE	BODY-WEIGHT WITHOUT FOOD	AVERAGE CARBON-DIOXIDE PRODUCED IN 4 HOURS CALCULATED ON A 7-KILOGRAM BASIS OF BODY-WEIGHT
1912	<i>kilos</i>	<i>grams</i>
Nov. 9	7.12	15.6
11	6.67	18.8
16	6.54	16.6
19	6.64	18.1
22	6.30	17.0
Average.....		17.2

TABLE 13.

*Carbon-dioxide production in experiments with food (750 grams of meat) with the dog "Flora."*

(Calculated to basis of 7 kilos of body-weight.)

DATE	BODY-WEIGHT WITHOUT FOOD	CARBON-DIOXIDE PRODUCTION IN 4-HOUR PERIODS					
		First period	Second period	Third period	Fourth period	Fifth period	Sixth period
1912	<i>kilos</i>	<i>grams</i>	<i>grams</i>	<i>grams</i>	<i>grams</i>	<i>grams</i>	<i>grams</i>
Oct. 25	7.6	26.5	26.2				
29	7.4	27.1	28.1				
Nov. 14	6.56	30.4					
20*	6.63				21.9		21.3
Average .....		28.0	27.2	27.2	21.9	21.9	21.3
Increment over fasting†		10.8	10.0	10.0	4.7	4.7	4.1
Total 24-hour increment				44.3			

\* Only 700 grams of meat were given on this day.

† For fasting value for 4 hours, i.e., 17.2 grams, see table 12.

4-hour period following the feeding of meat, with an average of 28.0 grams, an increment over the fasting value of 10.8 grams. For the second 4-hour period, we have an average of 27.2 grams or

an increment over the fasting value of 10.0 grams. No values were obtained for the third 4-hour period but, according to the usual custom, we have assumed the increment to be the same as in the preceding period, *i.e.*, 10.0 grams. In the fourth period, the increment was 4.7 grams, the same increment being assumed for the fifth period in which no observations were made. In the sixth and last period the increment was 4.1 per cent. These data show, therefore, that the base line was not reached even at the end of the twenty-four hours, there still being an increased carbon-dioxide production of about 4 grams above the normal. Computing the total increment in twenty-four hours as was done for the other dogs, we find it to be 44.3 grams. Since with a fasting value of 17.2 grams, the total carbon-dioxide output for twenty-four hours of a dog without food would be 103.2 grams, the increment of 44.3 grams after eating meat would be equal to approximately 43 per cent.

*Respiratory quotients obtained before and after the feeding of meat.*

It is obvious that the respiratory quotient, if exactly determined, is of value in these experiments even when the muscular activity in some of the periods was too great to admit of the results being used for comparison. These quotients were determined for a number of days on which the animal was without food, and are as follows:

October 21 .....	0.85	November 12.....	0.71
22.....	0.78	16.....	0.70
23.....	0.74	19.....	0.77
28.....	0.79	21.....	0.77
November 9.....	0.77	22.....	0.83
Average.....		0.77	

According to these fasting quotients, the dog was evidently not living wholly upon fat and inasmuch as the nitrogen excretion did not indicate an excessive disintegration of protein, we can only infer that the dog must have had a fairly liberal supply of glycogen which she could use when other food was not available. If the catabolism were of fat with a small proportion of protein, we should expect quotients averaging 0.75 or below, and the fact that the average is somewhat above this indicates a combustion of glycogen.

The respiratory quotients after feeding are of particular interest inasmuch as the study with this dog was made especially for determining the character of the catabolism following food. The quotients obtained under these conditions were as follows:

October 25.....	0.78	November 14.....	0.78
	0.71	15.....	0.79
29.....	0.80	20.....	0.79
			0.76

Aside from the second value for October 25, namely 0.71, it will be seen that all the quotients lie very close to 0.79 which is approximately the theoretical quotient for the combustion of protein; we may accordingly assume that for the greater part of the time during which these experiments were made, the dog was using protein. Even if the protein supply were deficient, the catabolism could not have been exclusively of fat, since this would tend to lower the quotient materially below 0.79, and the energy if not supplied by the combustion of protein must have been furnished to an appreciable degree (at least 30 per cent) by carbohydrates.<sup>23</sup>

The series of experiments with the dog "Flora," therefore, establishes the fact that the character of the catabolism after feeding, as shown by the respiratory quotient, was essentially the same as that indicated by the carbon-dioxide production of the normal dog "Clara," i.e., the combustion was in large part of protein, the balance of the energy undoubtedly being derived in part from fat and in part from glycogen. If, however, the dogs operated on had had a small storage of glycogen, as might naturally have been expected from their emaciated condition, the combustion would have been in part of protein and in large part of fat, so that the calorific equivalent of the carbon dioxide produced would be different; consequently, the values obtained in the experiments with the dogs operated on would not be comparable with those secured with the normal dog. Since, however, the respiratory quotients obtained in the experiments with "Flora" show that the character of the catabolism was unchanged by the operation, it is reasonable to believe that for purposes of comparison, the carbon-dioxide production may properly be taken as an index of the total catabolism in all of the experiments with the four dogs.

<sup>23</sup> Magnus-Levy: *Physiologie des Stoffwechsels*, in *von Noorden's Handbuch der Pathologie des Stoffwechsels*, i, p. 207. 1904-5.

*Physical condition during observation.* The dog was sick for a week following the operation on March 29, 1912, and ate but little. There was no glycosuria. On April 11, 1912, it was noted in the records that the dog was very active. A stool passed that day contained a large number of muscle fibers. By May 20, 1912, the weight had fallen to 8 kgms., and on July 22, 1912, it was down to 6.66 kgms. The limit of tolerance for glucose had fallen from above 100 grams to less than 40 grams by June 26, 1912. On September 6, 1912, the weight reached the lowest point, i.e., 6.13 kgms. A week later the feeding of fresh pig's pancreas was begun. The dog gained rapidly in weight, and when the feeding of pancreas was discontinued on October 21, 1912, she weighed 8.35 kgms. During the week that the respiration experiments were made, no pancreas was fed. On January 19, 1913, her weight was 7.15 kgms. Previous to this date she had been well and active, but when the dog was being taken from the weighing room to the roof, her head was crushed by the elevator. There was profuse hemorrhage from the mouth and she died almost instantly.

*Results of autopsy.* An autopsy was performed thirty minutes after the dog's death. There was some subcutaneous fat, the mesentery also containing a fair amount of fat. The scar of the operation showed only a very little thickening. The peritoneal cavity was dry and the serosa pale and glistening. About 6 cm. below the pylorus there were some adhesions between the duodenum and the adjacent loops of intestine, and some thin fibrous bands united the duodenum to the liver and the gall bladder. No tissue that could be definitely recognized as the pancreatic gland was found. At the site of the processus lienalis, near some large blood vessels that ran transversely in the mesentery, were small semi-opaque white nodules 1 to 2 mm. in size resembling fat tissue, but of firmer consistency, which formed a thin arborescent structure situated between the layers of the mesentery.<sup>24</sup> When the mesentery which extended from the duodenum to the splenic region was held to the light, this structure was brought plainly to view, and was found to be about 4 cm. long, 0.5 to 3 cm. in width, and not over 1 mm. in thickness. Nothing that resembled a duct was seen. This tissue could be traced to the wall of the duodenum where a suture surrounded by a little dense fibrous tissue 2 to 3 mm. thick was found. There was no trace of the corpus pancreatis. Near the former junction of the corpus pancreatis and the processus uncinatus was another silk suture in a small nodule of fibrous tissue. The duodenum was here bound to the large intestine by adhesions. Careful dissection failed to reveal any remains of the processus uncinatus, and none were found on histological examination.

#### *Nitrogen in the urine excreted by the dogs.*

During the research an attempt was made to collect the urine excreted in 24-hour periods and determine the nitrogen. Great difficulty was experienced in securing this separation, so that we

<sup>24</sup> On histological examination these nodules were found to have the structure of islands of Langerhans.

have no exact knowledge regarding the amounts. Furthermore, as the feces were voluminous and soft, there was undoubtedly at times contamination from this source. The determinations of the nitrogen excretion, which were made only for "Zep," "Pat," and "Flora," have no value other than to give the general impression that the dogs were not destroying an excessive amount of body-protein, which was fully confirmed by the series of experiments with "Flora" on days when she was regularly catheterized.

That the dogs did not store protein in the body after the ingestion of 750 grams of meat is shown by the 4-day absorption experiment with "Flora," November 12-15, 1912, in which she was given this amount of meat daily. The nitrogen in the food was 83.31 grams. The excretion of nitrogen in the feces was 35.18 grams, and in the urine, 48.15 grams, or a total excretion of 83.33 grams. There was thus a loss from the body of 0.02 gram of protein.

No great storage of protein was shown even when 1000 grams of meat were given daily. In a 3-day absorption experiment, May 24-26, 1911, "Zep" excreted in the feces 37.24 grams of nitrogen and in the urine, 44.42 grams, or a total excretion of 81.66 grams. The nitrogen given in the food was 84.84 grams, so that in this length of time the dog retained but 3.18 grams of nitrogen.

#### *Discussion of results.\**

*Gross metabolism of dogs without food.* The average carbon-dioxide excretion of the dogs used in this research, when without food and muscularly at rest, was as follows:

	Grams of carbon dioxide in 24 hours.
"Zep" .....	122
"Pat" .....	126
"Clara" .....	122
"Flora" <sup>25</sup> .....	103

<sup>25</sup> The determinations were made with the small apparatus. For purposes of comparison several experiments without food in which the small apparatus was used were also made with the normal dog "Clara," although the results are not included in this publication. The average 24-hour production of carbon dioxide, as obtained from the results of these selected half-hour periods, computed on the basis of 4 hours and 7 kgms. of body-weight, was 118 grams. A careful inspection of the kymograph records

Although these values were all computed to a uniform basis of 7 kgms. of body-weight, they do not materially differ from the actual measurements as the weights of the dogs were in all cases not far from 7 kgms. The exceptionally low value of 94 grams for "Zep" on July 14-15 was not included in making this average as we have reason to believe that so low a value was distinctly abnormal for this dog. These average values show that the total metabolism was not abnormal with either "Pat" or "Zep" as they agree remarkably well with that obtained for the normal dog "Clara."

From the determinations of the respiratory quotient, and other considerations which have been outlined in the discussion of these quotients, it can be seen that the carbon-dioxide production may be logically taken as an index of the total catabolism. Using the calorific value of carbon dioxide with a respiratory quotient of 0.79 as equal to 3.0 calories per gram, we may compute approximately the total heat output by multiplying by 3 the weight of carbon dioxide produced. That this method of computation may properly be used for purposes of comparison is shown by the fasting values obtained by Williams, Riche, and Lusk<sup>28</sup> in the direct heat measurements recently made on dogs, since we find that the total carbon-dioxide production when multiplied by 3 gives almost exactly the calories found. As a matter of fact, the dog used by the investigators cited had on at least three of the five days, a respiratory quotient considerably lower than those commonly found by us with our dog "Flora," but it is probable that this value of 3 calories per gram of carbon dioxide is not far from correct and hence it is justifiable to use it in this connection. Multiplying the weight of carbon dioxide by the calorific equivalent of carbon dioxide under these conditions, namely, 3 calories per gram, we then have for our dogs the following calorie values:

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shows that there was essentially the same muscular activity in the small as in the large chamber, and the measurement of the metabolism was on an average essentially the same for the 24-hour period. On the other hand, the dog "Flora" showed through all experiments a much lower muscular activity than did the dog "Clara," which easily accounts for her lower metabolism when compared with the other dogs. In fact, the records for the dog "Flora" show strikingly a much greater degree of muscular rest than any of the other dogs experimented with.

<sup>28</sup> Williams, Riche, and Lusk: this *Journal*, xii, p. 358, 1912, table 1.

	PER 24 HOURS	PER KILOGRAM PER 24 HOURS
	<i>calories</i>	<i>calories</i>
"Zep" .....	366	52
"Pat" .....	378	54
"Clara" .....	366	52
"Flora" .....	309	44

The value obtained for "Flora" is considerably lower than that for "Zep," "Pat," or "Clara," but we should emphasize the fact in this connection that the temperatures at which the measurements were made with "Flora" were invariably 2° or 3°C. higher than those used with "Zep" or "Pat," and the computations are based on *selected half-hour periods*.

It is of interest here to compare the results obtained on two dogs in Rubner's laboratory. According to Rubner's computations, one dog weighing 6 kgms<sup>27</sup> had a calorie output, fasting, of 51 to 58 calories per kilogram in twenty-four hours. For another dog weighing 5 kgms., Rubner<sup>28</sup> computed the fasting metabolism as 56 calories per kilogram.

The conditions under which the measurements were made in the two investigations cited varied somewhat from those in our experiments. In three of the experiments reported by Williams, Riche, and Lusk, when the extraordinarily low average value of 38.3 calories per kilogram per twenty-four hours was found, the temperature of the environment was the same as that in our experiments, *i.e.*, 26° and 27°C., but the dog was asleep in the respiration chamber. In our experiments, however, none of the measurements were made exclusively during a period of sleep, and there was some minor muscular activity in all periods. Information regarding the muscular activity of Rubner's dogs is entirely lacking, but the statement is made that the temperatures at which the measurements were secured averaged always about 30° to 31°C.

While it is impossible to explain the differences in results, and they are undoubtedly caused by several factors, of which two are temperature difference and muscular activity, it is important in

<sup>27</sup> Rubner: *Die Gesetze des Energieverbrauchs bei der Ernährung*, Leipzig, 1902, pp. 42-46.

<sup>28</sup> Rubner: *loc. cit.*, pp. 318 and 323.



this connection only to note that the results obtained with our dogs come within what may be termed normal limits. The lower values found for "Flora" as compared with those obtained with "Zep," "Pat," and "Clara," are distinctly due to several facts, *i.e.*, that "Flora" was a remarkably quiet dog, quieter than any dog heretofore used in this laboratory as a subject; and that practically all of the experiments were of short duration and of selected periods of minimum muscular activity. The results are also in conformity with the values obtained with the normal dog "Clara" when the small respiration chamber was used, which were lower than those obtained with the larger chamber when the experiments lasted several hours and often the whole day. The experimental evidence having established the fact that "Clara" was a normal dog, the values obtained with her are used in this discussion as a base line.

*Comparison of the increments in the carbon-dioxide production following the feeding of meat to three dogs with pancreatic achylia and to a normal dog.* The fact that all three dogs after operation showed a reaction to the ingestion of meat did not by any means throw definite light upon the question at issue, namely, whether or not the increase in the metabolism following the ingestion of meat is due to the mechanical movement of the food along the intestinal tract, and it is only by comparing the data with those obtained with a normal dog that such information can be gained.

Having shown that the calorie value is proportional to the carbon dioxide, instead of expressing the increments in the form of calories, we may compare simply the percentages of increment in the carbon-dioxide output after the ingestion of meat. This comparison is made in table 14. Following the feeding of 500 grams of meat, the average percentage increase in the carbon-dioxide production was with "Zep," 17 per cent; with "Pat," 22 per cent; and with the normal dog "Clara," 28 per cent. After giving 750 grams of meat, there was an average increase with "Zep" of 48 per cent; with "Pat," 25 per cent; with "Flora," 43 per cent; and with the normal dog "Clara," 62 per cent. "Zep" was the only dog who was given 1000 grams of meat, the percentage increase in the carbon-dioxide production following the feeding of this large amount being 53 per cent. No similar experiments were made with the normal dog "Clara."

When these results are compared, it is at once seen that the increase in the carbon-dioxide production was much larger with the normal dog "Clara" than with the three dogs operated upon. This was particularly true when 750 grams of meat were fed, although also apparent when only 500 grams of meat were given. In fact, with "Clara" the increase following the feeding of 750 grams of meat was 9 per cent greater than the increase with "Zep" when given as large an amount as 1000 grams.

TABLE 14.

*Comparison of the 24-hour increments in the carbon-dioxide production after feeding meat to "Zep," "Pat," and "Flora," and the normal dog "Clara."*  
(On the basis of 7 kgms. of body-weight.)

	"ZEP"	"PAT"	"FLORA"	"CLARA" (normal)
Fasting value (grams) . . . . .	122	126	103	122
Increase following feeding of 500 grams of meat (per cent).	20 16 13 16 16 20	17 25 23		31 26 28
Average . . . . .	17	22		28
Increase following feeding of 750 grams of meat (per cent)	39 56	25	43	62
Average . . . . .	48	25	43	62
Increase following feeding of 1000 grams of meat (per cent) . . . . .	53			

If the increase in the metabolism following the ingestion of meat were due to nutrients absorbed out of the food and carried to the blood cells, we should expect a lower increment in the metabolism of the dogs operated upon than with the normal dog, since so large a proportion of the food was not absorbed. Among the possible objections that may be raised to these experiments, and we recognize that many objections can be raised to them if they are to be used in any other manner than was here intended, it may

be reasoned that dogs in so emaciated a condition might rapidly store the protein ingested instead of burning it. Under these conditions, Rubner has shown that the storage of protein does not exercise an influence upon the heat production. As a matter of fact, however, the nitrogen balance experiments that have been made with these dogs have shown invariably either a nitrogen equilibrium or very slight gains or losses, indicating that in general the dogs are in nitrogen equilibrium. Certainly no evidence was obtained to show that there was a large storage of protein during the ingestion of meat, so that the lower metabolism obtained with the abnormal dogs could not have been due to this factor.

On the other hand, if the increase following the ingestion of meat were due in large part to mechanical action, we should expect much greater increases in the carbon-dioxide output with the dogs operated upon than with the normal dogs. The stools voided by these dogs were enormous and, according to this latter theory, the mechanical work required to pass the mass of unabsorbed food through the intestinal tract would result in an increased metabolism, unless compensated by some other powerful factor. This is not shown, however, to be the case as the metabolism was considerably lower under these conditions than with the normal dogs.

It may be argued that with the dogs operated on, one of the largest glands in the body—the pancreas—had ceased to act, and if glandular work plays a large rôle in increasing the metabolism after the ingestion of food, this absence of the pancreatic function would lead to a somewhat decreased metabolism. Glandular work cannot be estimated by our method and hence the results should be considered with this in mind. It would be necessary, however, to ascribe to the work of the pancreas an excessive influence upon the metabolism if its absence were not only to compensate for the large increase in the intestinal activity and supposed incidental heat production, but actually to depress the metabolism below the normal level.

The results of these experiments show that there is no large energy transformation incidental to segmentation, peristalsis, glandular activity of stomach, liver and intestine, and the movement of the unabsorbed food through the intestinal tract. The attempt to explain the increased metabolism following the ingestion of food by the theory that the increase is a consequence of such movements is, therefore, not justifiable.



# THE INFLUENCE OF AGE AND OF DIET ON THE RELATIVE PROPORTIONS OF SERUM PROTEINS IN RABBITS.

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(From the Rudolph Spreckels Physiological Laboratory of the University of California.)

(Received for publication, April 29, 1913.)

In carrying out the following analyses, I employed the method devised by Robertson<sup>1</sup> for the determination of the serum proteins by the use of the refractometer.

## A. *Experiments on rabbits of different ages.*

The rabbits used in these experiments were bred from six females (presumably Belgians), and a black "Flemish Giant" buck. As far as possible, the conditions were kept constant throughout the six months over which the experiments extended. The rabbits were fed on grain and alfalfa hay. Before bleeding, the animals were deprived of food for twenty-four hours and of water for two hours at least. They were bled by cutting the carotid of one side, care being taken not to sever the trachea and oesophagus. The blood was received into a beaker, and defibrinated by shaking with glass beads; then immediately centrifuged for one hour and forty-five minutes. To prevent evaporation as far as possible, all the Erlenmeyer flasks used were provided with rubber stoppers, and when filtering, the funnels were covered with moistened filter papers.

The results of the analyses are given in Table I. A comparison of the figures indicates the following:

1. The percentage of total proteins, and the percentage of total albumins show a slight gradual increase with age, from 21 to 140 days. The last three experiments, including the two adult females, show again a decrease in the percentages. It should be noted that

<sup>1</sup> T. Brailsford Robertson: this *Journal*, xiii, p. 325, 1912.

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TABLE I.

RABBIT NUMBER	AGE	WEIGHT*	"INSOL- UBLE" GLOBU- LINS	PERCENTAGE RATIO TO TOTAL PROTEINS					
				TOTAL GLOBU- LINS	TOTAL ALBU- MINS	TOTAL PRO- TEINS	"Insol- uble" globu- lins	Total globu- lins	Total albu- mins
				per cent	per cent	per cent	per cent		
	days	grams	per cent	per cent	per cent	per cent	per cent		
			±0.04	±0.15	±0.2	±0.2	±0.4	±2	±2
1	21	180(5)	0.21	1.1	4.4	5.5	3.8	20	80
2	24	183(4)	0.21	1.0	4.6	5.6	3.8	18	82
3	25	355(4)	0.21	0.7	4.6	5.3	2.6	13	87
4	30	433(4)	0.28	0.9	5.1	6.0	4.7	15	85
5	32	206(4)	0.24	0.7	5.0	5.7	4.2	13	87
6	39	807(2)	0.27	1.4	4.8	6.2	4.4	23	77
7	39	405(4)	0.28	0.7	5.1	5.8	4.8	12	88
8	54	692(3)	0.31	1.2	5.6	6.8	4.6	18	82
9	54	985(2)	0.31	1.2	5.0	6.2	5.0	19	81
10	57	1215(2)	0.24	1.5	4.8	6.3	3.8	24	76
11	57	1156	0.31	1.7	5.4	7.1	4.4	24	76
12	87	1656	0.35	1.1	6.0	7.1	4.9	16	84
13	87	1475	0.34	1.9	5.2	7.1	4.8	27	73
14	87	1339	0.34	2.2	5.2	7.4	4.6	29	71
15	87	1694	0.28	2.1	5.4	7.5	3.7	28	72
16	87	1890	0.39	0.9	5.8	6.7	5.8	13	87
17	97	2020	0.34	1.5	5.3	6.8	5.0	22	78
18	97	2024	0.31	1.6	5.3	6.9	4.5	23	77
19	98	1812	0.24	1.6	4.8	6.4	3.7	25	75
20	98	1747	0.34	1.8	5.6	7.4	4.6	24	76
21	102	1637	0.28	1.9	5.2	7.1	3.9	27	73
22	109	1836	0.24	1.3	5.2	6.5	3.7	20	80
23	115	2118	0.21	1.5	5.6	7.1	2.9	21	79
24	115	2333	0.21	1.5	6.1	7.6	2.7	20	80
25	125	2286	0.27	1.7	5.8	7.5	3.6	23	77
26	125	2323	0.27	1.5	6.0	7.5	3.6	20	80
27	136	2035	0.28	1.6	6.4	8.0	3.5	20	80
28	138	2465	0.27	1.3	6.6	7.9	3.4	17	83
29	138	2375	0.24	1.7	7.1	8.8	2.7	19	81
30	151	2065	0.31	2.1	5.4	7.5	4.1	28	72
31	151	2630	0.28	1.8	5.8	7.6	3.7	24	76
32	158	3030	0.28	1.6	5.3	6.9	4.1	23	77
†33	(1 Yr.?)	3130	0.24	1.6	5.0	6.6	3.6	24	76
†34	(1 Yr.?)	2495	0.28	1.2	5.2	6.4	4.3	19	81
†35	(1 Yr.?)	2950	0.42	3.1	4.2	7.3	5.7	43	57

\*The numbers in the parentheses in the column of weights indicate the number of rabbits used in the experiment, the weight given being an average of the total weights.

†The rabbits used in these experiments were adult females, and were suckling young at the time; the young were used in experiments 1 and 2, above.

‡This rabbit was also one of the adult females used for breeding. On autopsy, she was found to have an abscess of the uterus, the organ being enormously distended so as to nearly half fill the abdominal cavity and containing the necrotic remains of three embryos.

Reiss<sup>2</sup> has also found that in human beings the concentration of the total proteins in the serum of sucklings is lower than it is in the serum of adults.

2. The percentage of total globulins also shows a slight increase, but with greater individual variations.

3. The percentage of the "insoluble" globulins shows no constant variation corresponding with the age of the animals.

4. Considering the ratios which the various proteins bear to the total protein content, we note the following: *a.* There is no correspondence between the ages of the animals and the variations of the relative proportions of the three groups of proteins.

*b.* Taking the averages of the figures in the last three columns of Table I, we have for the series:

"Insoluble" globulins.....	4.0	$\left\{ \begin{array}{c} 5.8 \\ 2.7 \end{array} \right\}$	( $\pm 0.4$ )
Total globulins.....	21.0	$\left\{ \begin{array}{c} 29.0 \\ 12.0 \end{array} \right\}$	( $\pm 2.0$ )
Total albumins.....	79.0	$\left\{ \begin{array}{c} 88.0 \\ 71.0 \end{array} \right\}$	( $\pm 2.0$ )

The first figure opposite each group represents the average percentages; the figures in brackets the highest and lowest observed in any individual; and the figures following the plus and minus signs, the experimental error.

The average figures here are intermediate between those which Robertson<sup>3</sup> gives for "normal" rabbits, and for rabbits which had fasted five days, but the variation between the highest and lowest percentages is greater; this is probably due to the fact that I have analyzed the sera of a much larger number of animals, and hence have encountered more instances of extreme variation from the mean.

*c.* It is of interest to observe that the proportion of globulin to albumins in the serum of the animal (No. 35) with the uterine abscess was enormously greater than that observed in any of the normal animals, although the percentage of total proteins was normal.

<sup>2</sup> E. Reiss: *Jahrb. f. Kinderheilk.*, lxx, Heft 3, 1909.

<sup>3</sup> T. Brailsford Robertson: *loc. cit.*

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### *B. Experiments on the effect of diet.*

Six rabbits were fed on milk alone for a period of two weeks, a control series being fed on a mixed diet of grain and alfalfa hay during the same time. The rabbits used were of medium size. They were bled, and the blood centrifuged and analyzed, as in the preceding experiments. The milk-fed rabbits were very emaciated; they were found to have lost in weight an average of about 200 grams (partly to be accounted for by the fact that the stomach and intestine were practically empty).

TABLE II.  
*Rabbits fed on milk.*

RABBIT NUMBER	"INSOL- UBLE" GLOBULINS	TOTAL GLOBULINS	TOTAL ALBUMINS	TOTAL PROTEINS	PERCENTAGE RATIO TO TOTAL PROTEINS		
					"Insoluble" globulins	Total globulins	Total albu- mins
	per cent	per cent	per cent	per cent			
	≈0.04	≈0.15	≈0.2	≈0.2	≈0.4	≈2	≈2
1	0.24	2.0	5.2	7.2	3.3	28	72
2	0.35	2.1	4.8	6.9	3.6	30	70
3	0.20	1.4	5.2	6.6	3.0	21	79
4	0.20	1.4	5.2	6.6	3.0	21	79
5	0.34	1.6	4.8	6.4	5.3	25	75
6	0.34	1.5	6.0	7.5	4.5	20	80
Average	0.26	1.7	5.8	6.9	3.6	24	76

*Control series.*

1	0.21	1.7	5.0	6.7	3.1	24	75
2	0.24	1.6	5.0	6.6	3.6	24	76
3	0.14	1.3	4.6	5.9	2.4	22	78
4	0.14	0.8	5.4	6.2	2.3	13	87
5	0.24	0.8	5.0	5.8	4.1	14	86
6	0.27	1.2	5.2	6.4	4.2	19	81
Average	0.22	1.2	5.0	6.3	3.3	19	81

Comparison of the results from the two series (see Table II), shows the following:

1. The average percentages of the total proteins, as well as of each group, is higher in the case of the milk-fed rabbits. This is



probably to be accounted for by the fact that during the last few days the animals did not drink much of the milk, and were in a fasting condition.<sup>4</sup>

2. The milk-fed animals show an increase in the relative amount of the globulins, over the control series; but this difference is only slightly more than twice the experimental error of the determination.

#### CONCLUSIONS.

1. The percentage of total proteins in the blood serum of rabbits increases with age between the ages of 21 to 140 days. Fully adult animals have a slightly lower content of proteins in their blood serum than animals which are between 100 and 150 days old.

2. The relative proportion of "insoluble" globulins, "soluble" globulins, and albumins in the blood serum of rabbits varies somewhat in different individuals (fasted for 24 hours). There is, however, no correspondence between the ages of the animals and the variations in the relative proportions of these proteins.

3. Adult (medium-sized) rabbits fed upon a diet composed exclusively of milk for a period of two weeks, yield serum containing a slightly higher percentage of proteins than rabbits fed for a like period upon a normal mixed diet of grain and alfalfa; but the relative proportions of the above-mentioned proteins in the serum of the milk-fed animals did not differ in any significant degree from the proportions observed in the normal animals.

4. One animal which exhibited a pathological condition (uterine abscess) yielded serum which contained twice the relative proportion of globulins observed in normal animals, although the percentage of total proteins in the serum of this animal was normal.

<sup>4</sup> Cf. T. Brailsford Robertson: *loc. cit.*



## TOXIC BASES IN THE URINE OF PARATHYROIDECTOMIZED DOGS.

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(Received for publication, April 30, 1913.)

In a recent paper<sup>1</sup> I demonstrated the presence of methyl guanidine in the urine of a parathyroidectomized dog, and called attention to the presence of some other bases. It is the purpose of this paper to report the occurrence of methyl guanidine in the urines of each of five other parathyroidectomized dogs, and to describe some of the other bases more fully. Experiments have also been made to determine in what manner mercuric chloride might best be used as a precipitant for their isolation.

Since the isolation of toxic bases from a urine involves a large number of manipulations and often large precipitates, quantitative results are hardly to be expected. The urines were therefore run in pairs, and the same technique so far as possible applied to each. I hoped in this way to be able to attribute any pronounced differences in the bases of two urines, similarly examined, to variations in metabolism. Moreover, substances that agree in a number of their properties when present in small quantities in each urine, could be combined.

The urines of the first two parathyroidectomized dogs were treated separately and similarly as follows: After each micturition the samples, collected by drainage from the cage, were acidified with HCl to precipitate the kynurenic acid. After standing in the cold until complete precipitation occurred, the precipitate was filtered off and the filtrate treated with tannin, barium hydroxide, sulphuric acid, and lead oxide according to Kutscher's method<sup>2</sup> for

<sup>1</sup> *This Journal*, xii, p. 313, 1912.

<sup>2</sup> *Zeitschr. f. physiol. Chem.*, xlviii, p. 1, 1906.

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the removal of proteins. The urines thus purified were treated with mercuric chloride and sodium acetate according to the method of Engeland.<sup>3</sup> The mercury salts of the bases thus obtained were taken up in dilute hydrochloric acid, decomposed with hydrogen sulphide, and the mercuric sulphide filtered off. The filtrate containing the hydrochlorides of the bases was evaporated to a syrup, and the organic substances extracted with methyl alcohol and the insoluble portion filtered off. After evaporation of the methyl alcohol, those substances easily soluble in ethyl alcohol were taken up in this solvent and treated with an alcoholic solution of platinum chloride. The precipitate that formed was filtered off, taken up in hot water and decomposed with hydrogen sulphide. The platinum sulphide was filtered off, and the filtrate concentrated and treated with gold chloride (Fraction A). Likewise the platinic filtrate after evaporation of the alcohol was decomposed with hydrogen sulphide and the platinum sulphide filtered off. The aqueous solution thus obtained was concentrated and treated with gold chloride (Fraction B). Those substances difficultly soluble in alcohol were taken up in water and treated with absolute alcohol until no more precipitate formed. The precipitate was filtered off, taken up in water and treated with picrolonic acid in aqueous solution (Fraction C). The filtrate after removal of the alcohol was likewise treated with picrolonic acid (Fraction D).

From fraction A of urine 1, 0.7 gram of yellow needles melting after two recrystallizations at 200°C. was obtained. They appeared similar to those mentioned in my preliminary report. From urine 2, 0.5 gram of the similar needles melting after two recrystallizations at from 200° to 205°C. was obtained. A number of crystals from each sample intimately ground together became soft at 200°C. and did not melt until 206°C. They were very insoluble in cold water and not readily soluble in hot water. After the removal of the gold each gave the diazo reaction with diazobenzene-sulphonic acid and sodium carbonate. A weighed portion of the combined crystals was taken up in water and the gold removed as the sulphide. This was converted into free gold and weighed. The filtrate containing the bases was treated with picrolonic acid.

<sup>3</sup> *Zeitschr. f. physiol. Chem.*, lvii, p. 49, 1908.

The precipitate thus obtained after recrystallization melted at 266°C.<sup>4</sup>

*The gold salt.*

0.4301 gm. gave 0.2132 gm. Au.  
 0.6008 gm. gave 0.2997 gm. Au.  
 0.4453 gm. at 21°C. and 732.5 mm. gave 23 cc. N.

*The picrolonate.*

0.2000 gm. at 22.5°C. and 739 mm. gave 43.8 cc. N.  
 0.1609 gm. gave 0.2762 gm. CO<sub>2</sub> and 0.0576 gm. H<sub>2</sub>O.

	Calculated for C <sub>6</sub> H <sub>5</sub> N <sub>3</sub> ·2(AuCl <sub>4</sub> ):	Found:	
		a	b
Au.....	49.9	49.67	49.88
N.....	5.32	5.68	

	Calculated for C <sub>6</sub> H <sub>5</sub> N <sub>3</sub> ·2(C <sub>10</sub> H <sub>8</sub> N <sub>4</sub> O <sub>4</sub> ):	Found:	
		a	b
N.....	24.14		24.13
C.....	46.92		46.81
H.....	3.91		4.01

This substance appears from the diazo reaction, the melting points, and analyses, to be  $\beta$ -imidazolyethylamine. I am surprised at its presence among the substances readily soluble in alcohol since the hydrochloride of this substance is described as being sparingly soluble in alcohol.<sup>5</sup>

There next occurred in each urine fraction, a crop of rhomboid plates. They were recrystallized twice, and melted slowly between 241° and 245°C. Portions of the crystals from each urine were intimately mixed. These melted at 243°C. After another recrystallization their form changed to needles but the melting point was not altered. From each urine 0.3 gram was obtained.

From urine 1, 0.2259 gm. substance gave 0.1019 gm. Au.

From urine 2, 0.1845 gm. substance gave 0.0831 gm. Au.

	Calculated for C <sub>6</sub> H <sub>5</sub> NOCl·AuCl <sub>3</sub> :	Found:	
		a	b
Au.....	44.52	45.16	45.04

<sup>4</sup> Thermometer readings are not corrected. The nitrogen was collected over 40 per cent KOH; barometer readings are taken from an instrument with a glass scale, reading directly; direct readings are given not corrected except where indicated.

<sup>5</sup> *Zeitschr. f. physiol. Chem.*, lxx, p. 504, 1910.

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The melting point is that of choline aurichloride and the gold content indicates a substance of equal molecular weight.

After the solutions became quite concentrated, a large quantity of needles and some plates separated out. After three recrystallizations they began to soften in the melting tube at 262°C., became almost fluid at 286°C. and melted rising in the tube with bubble formation at 292°C. to 296°C. On analysis the substance was found to be free from carbon and proved to be the gold salt of ammonium chloride.

Fractions B of urines 1 and 2, the filtrates from the platinum precipitates, gave after concentrating and treatment with gold chloride, each, a precipitate of yellow needles. These after one recrystallization melted sharply at 198°C. Urine 1 gave 2.3 grams; urine 2 gave 2.9 grams. They were more soluble in alcohol and ether than in water. The gold was removed from a portion of the united needles, and weighed; the filtrate was treated with picrolonic acid. The picrolonate melted sharply at 275°C. with decomposition and bubble formation.

### *The gold salt.*

0.1602 gm. gave 0.0763 gm. Au.

### *The picrolonate.*

0.2023 gm. at 22.5°C. and 745 mm. gave 53.5 cc. N.

0.0950 gm. gave 0.1490 gm. CO<sub>2</sub> and 0.0369 gm. H<sub>2</sub>O.

	Calculated for C <sub>2</sub> H <sub>5</sub> N <sub>2</sub> (AuCl <sub>4</sub> ):	Found:
Au .....	47.7	47.6
	Calculated for C <sub>3</sub> H <sub>7</sub> N <sub>3</sub> (C <sub>10</sub> H <sub>8</sub> N <sub>4</sub> O <sub>3</sub> ):	Found:
N .....	29.14	29.31
C .....	42.7	42.78
H .....	4.45	4.35

This substance is evidently methyl guanidine as shown by the properties and analyses of these derivatives. After no more methyl guanidine aurochloride separated out, the gold was removed as the sulphide from the mother solution and the filtrates united and treated with picrolonic acid. A dark red, very insoluble precipitate formed. By burning in a crucible it left magnetic iron oxide. A voluminous precipitate was next obtained which melted after

two recrystallizations at 346°C. The yield was slightly over 1 gram.

0.1126 gm. substance at 23.5°C. and 738 mm. gave 22.2 cc. N.

0.1129 gm. substance at 16.25°C. and 745 mm. gave 21.0 cc. N.

0.1439 gm. substance gave 0.2327 gm. CO<sub>2</sub> and 0.042 gm. H<sub>2</sub>O.

	Found:	
	<i>a</i>	<i>b</i>
N.....	21.54	21.21
C.....	44.1	44.14
H.....	3.27	3.49

On burning in a porcelain crucible the substance emitted a strong peach-blossom odor and left no ash. It needs further study.

Fraction C, the alcoholic precipitate of the substances difficultly soluble in alcohol, contained a large quantity of inorganic salts. However from this fraction of urine 2 a picrolonate was obtained. After several recrystallizations, it darkened at 340°C. and did not melt below 360°C. The yield was about 2 grams.

0.1912 gm. substance at 22.1°C. and 741 mm. gave 35.1 cc. N.

0.1713 gm. substance at 23.5°C. and 740.5 mm. gave 31.2 cc. N.

0.2347 gm. substance gave 0.0644 gm. H<sub>2</sub>O and 0.3704 gm. CO<sub>2</sub>.

0.1822 gm. substance gave 0.0530 gm. H<sub>2</sub>O and 0.2847 gm. CO<sub>2</sub>.

	Found:	
	<i>a</i>	<i>b</i>
N.....	20.25	20.04
C.....	42.9	42.6
H.....	3.07	3.25

This substance has about the same solubility as the picrolonate obtained by treating ammonium chloride with picrolonic acid. The latter however melts at 278°–280°C.

Fraction D gave a small precipitate with picrolonic acid which upon an attempt at recrystallization did not precipitate until the solution had almost evaporated to dryness. It cannot be studied until more substance is at hand.

The urines obtained from dogs 3 and 4 were treated similarly to those of dogs 1 and 2, with the exception that the removal of the proteins was omitted. After the removal of kynurenic acid, the urines were evaporated to a syrup, taken up in methyl alcohol,

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filtered and again evaporated to remove the excess of HCl, and then taken up in water. This solution was treated with mercuric chloride and sodium acetate according to Engeland's method to precipitate the bases. The urine fractions were similar to those of urines 1 and 2. Fractions A are again the platinum precipitates of substances soluble in alcohol. Fraction B of each urine is the filtrate from this platinum precipitate. Fraction C is the alcoholic precipitate of substances insoluble in alcohol. Fraction D, the filtrate from this precipitate.

Fraction A of urine 2 upon concentration and treatment with gold chloride gave 0.6 gram of rhomboid plates which after two recrystallizations melted at 248°C.

0.1901 gm. substance gave 0.085 gm. Au.

	Calculated for $C_{14}H_{14}NO AuCl_2$ :	Found:
Au.....	44.52	44.7

From urine 4 only a few needles melting at 238°C. were obtained. The quantity was too small for purification and analyses. As the solutions concentrated a large quantity of the aurochloride of ammonia was obtained.

Fractions B, the filtrates from the platinum precipitates, were found to reduce a test portion of gold chloride. After study they revealed the presence of ferrous iron. They were therefore made alkaline with  $Ag_2O$ . ( $Ag_2O$  was used instead of other alkalis because it could be removed so readily.) The precipitate obtained was decomposed with  $H_2S$  and gave upon removal of the silver sulphide, acidifying with HCl and concentrating, a large crop of beautiful translucent green rhomboid plates. These were very soluble in water and alcohol, and gave with potassium ferricyanide, the reaction for ferrous iron. They contained a large quantity of water of crystallization. They were dried by standing in a desiccator over  $H_2SO_4$  for several days. In a vacuum tube under a slow stream of dry air and at 120°C. they lost after one-half hour a portion of their water of crystallization.

2.0298 gms. substance lost 0.3611 gm.  $H_2O$  at 120°C.

0.2729 gm. substance gave 0.1350 gm.  $AgCl$ .

0.3500 gm. substance gave 0.1728 gm.  $Fe_2O_3$ .

	Calculated for $FeCl_2$ :	Found:
Fe.....	44.08	34.53
Cl.. ..	55.92	45.8



Evidently all of the water of crystallization was not lost by the drying. The substance appears to be ferrous chloride. The form in which the iron was excreted into the urine is not known, but it appears that it might, in part, have been part of a protein molecule since urines 1 and 2, from which the proteins were removed, contained much less iron.

The filtrates from the silver precipitates, after the removal of the silver, gave upon treatment with gold chloride crops of beautiful needles. These after recrystallization melted sharply at 198°C. They were soluble in water and more readily soluble in alcohol and ether. Urine 3 gave 2.2 grams, urine 4 gave 1.7 grams. After recrystallizing from alcohol ether and water, a portion was freed from gold and converted into the picrolonate. This melted sharply, rising in the tube at 275°C. with decomposition instead of at about 270° as described by Achelis.

*The gold salt.*

0.1031 gms. gave 0.4930 gms. Au.

*The picrolonate.*

0.1024 gms. at 25°C. and 742 mm. gave 27.4 cc. N.

	Calculated for $C_2H_4N_2(AuCl_4)$ :	Found:
Au .....	47.7	47.81
	Calculated for $C_3H_7N_2(C_{10}H_8N_4O_6)$ :	Found:
N .....	29.14	29.29

Their solubilities and melting point, the analyses, and the urine fraction from which they were obtained, characterize them as the gold salts of methyl guanidine. These crystals were followed in urine 4 by a small quantity of cubes and short rectangular prisms of a brown color. The quantity was too small for purification. The mother solutions from both urine fractions were united and the gold removed with hydrogen sulphide. The filtrates thus obtained were treated with picrolonic acid. About 0.8 gram of a substance precipitated which after two recrystallizations melted at 260°C. The substance was quite insoluble in water.

0.1783 gm. substance at 22.0°C. and 748.5 mm. gave 44.2 cc. N.

	Calculated for $C_3H_7N_2(C_{10}H_8N_4O_6)$ :	Found:
N .....	27.97	27.73

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This substance agrees in melting point and nitrogen content with symmetrical dimethyl guanidine.

Fraction C, the alcoholic precipitate of substances insoluble in alcohol, gave with picrolonic acid a precipitate which after several recrystallizations exploded at 348°C.

0.0752 gm. substance at 23°C. and 732 mm. gave 14.9 cc. N.

0.1619 gm. substance gave 0.2474 gm. CO<sub>2</sub> and 0.0490 gm. H<sub>2</sub>O.

0.1070 gm. substance gave 0.1635 gm. CO<sub>2</sub> and 0.0364 gm. H<sub>2</sub>O.

	Found:	
	a	b
N .....	21.56	
C .....	41.7	41.74
H .....	3.43	3.81

This substance requires further study. It is not very insoluble in water and may not be pure. Other derivatives are being prepared.

*Urine 5.* In order to determine if the choline present in the preceding urines might have arisen from phosphatides in the urine, an attempt was made to isolate such a substance. The urine was therefore extracted with an equal volume of ether. Upon evaporation of the ether only a trace of a lipoid substance was found. This gave a precipitate with cadmium chloride, but was too small to examine further. The urine was then carefully neutralized, evaporated to a syrup and again extracted with ether. Upon evaporation of the ethereal extract no better result was obtained. Since the choline bases are known to give mercuric chloride salts which are less soluble in alkaline alcohol than in alkaline water, the syrupy residue of this urine was taken up in a small quantity of warm alcohol and the hot solution saturated with mercuric chloride and potassium acetate.

It was then treated with a hot saturated alcoholic solution of mercuric chloride and potassium acetate. While warm the mixture, in a wide mouthed bottle, was permitted to evaporate and then placed in the cold. When precipitation was complete, the precipitate was filtered off, taken up in dilute HCl, and decomposed with H<sub>2</sub>S. The mercury sulphide was filtered off and the filtrate strongly acidified with HCl. After standing two days the

kynurenic acid had all precipitated and was then filtered off. The filtrate was evaporated to a syrup. The residue was now extracted with methyl alcohol, filtered, the alcohol evaporated, and the residue again extracted with methyl alcohol. After another repetition of the process no more inorganic salts were present in the extract, with the exception of ammonium chloride. The methyl alcohol was removed and the residue taken up in ethyl alcohol. The alcohol was evaporated off and the process repeated until only those substances easily soluble in alcohol were dissolved. The solution was then treated with alcoholic platinum chloride and the precipitate, which formed, filtered off (Fraction A). The alcohol was evaporated from the filtrate, the residue taken up in hot water and the platinum removed as the sulphide. The filtrate thus obtained was concentrated and treated with gold chloride (Fraction B). As in the previous urines the substances not dissolved by the extracting alcohol were taken up in water and precipitated with alcohol. This gave a precipitate (Fraction C) and a filtrate (Fraction D).

Fraction A, the platinum precipitate, was taken up in hot water and the substances readily soluble filtered off, as division 1. Substances left undissolved were again extracted with hot water, and the solution filtered from the insoluble portion (division 2). Those substances still undissolved were suspended in hot water and the three divisions decomposed with  $H_2S$ . The platinum sulphide was filtered from each. Division 1 was treated with gold chloride and gave a precipitate of rhomboid plates weighing about 2 grams. After two recrystallizations it melted at  $238^\circ C$ . The gold was removed as the sulphide, converted into free gold and weighed. The filtrate from the gold sulphide upon treatment with picronic acid gave a precipitate. This after one recrystallization melted cloudy at  $178^\circ C$ . and decomposed at about  $230^\circ C$ . The substance was dried in a vacuum. At  $115^\circ C$ . it lost water of crystallization.

*Gold salt.*

0.7392 gm. substance gave 0.3393 gm. Au.

	Calculated for $C_8H_{12}N \cdot AuCl_3$	Found:
Au.....	46.4	45.9

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### *The picrolonate.*

0.0979 gm. substance at 15°C. and 748.5 mm. gave 17.2 cc. N.  
 0.2253 gm. substance at 21.5°C. and 740 mm. gave 40.8 cc. N.  
 0.2107 gm. substance gave 0.1140 gm. H<sub>2</sub>O and 0.3978 gm. CO<sub>2</sub>.  
 0.1630 gm. substance gave 0.0884 gm. H<sub>2</sub>O and 0.3088 gm. CO<sub>2</sub>.

	Calculated for C <sub>8</sub> H <sub>11</sub> N(C <sub>10</sub> H <sub>8</sub> N <sub>4</sub> O <sub>8</sub> ):	Found:	
		a	b
N.....	20.05	20.26	20.07
C.....	51.57	51.49	51.67
H.....	5.44	6.05	6.06

The substance is neurine as shown by the properties and analyses of the two derivatives.

The second platinic division was treated with picrolonic acid. It gave a precipitate weighing 2.1 grams which upon recrystallization was fractionated into two portions. The first and larger portion after recrystallizing several times melted between 284°C. and 286°C. The substance is quite insoluble in water.

0.1779 gm. substance at 22.5°C. and 740.8 mm. gave 41 cc. N.  
 0.1596 gm. substance at 22.0°C. and 742.0 mm. gave 36.8 cc. N.  
 0.1526 gm. substance gave 0.0609 gm. H<sub>2</sub>O and 0.2583 gm. CO<sub>2</sub>.  
 0.1715 gm. substance gave 0.0648 gm. H<sub>2</sub>O and 0.2895 gm. CO<sub>2</sub>.

	Calculated for C <sub>8</sub> H <sub>9</sub> N <sub>5</sub> O(C <sub>10</sub> H <sub>8</sub> N <sub>4</sub> O <sub>8</sub> ):	Found:	
		a	b
N.....	25.2	25.45	25.54
C.....	46.00	46.17	46.06
H.....	4.35	4.46	4.23

The second and smaller fraction after three recrystallizations softened and drew together in the melting tube at 240°C. and melted at 264°C.

0.0979 gm. substance at 18°C. and 736.5 mm. gave 22.5 cc. N.  
 0.1038 gm. substance gave 0.0391 gm. H<sub>2</sub>O and 0.1732 gm. CO<sub>2</sub>.

	Calculated C <sub>8</sub> H <sub>10</sub> N <sub>5</sub> 2(C <sub>10</sub> H <sub>8</sub> N <sub>4</sub> O <sub>8</sub> ):	Found:
N.....	25.74	25.85
C.....	45.84	45.51
H.....	3.97	4.21

These substances freed from picrolonic acid did not give the diazo reaction and their structure is unknown. When more material is obtainable they will be studied further.

The third division was treated with gold chloride. A large precipitate of needles was obtained. These after recrystallization melted at 292°C. and appear to be the gold salt of ammonium chloride. After the solution had become quite concentrated a small quantity of flat yellow needles was obtained. They were not identified.

Fraction B, the alcoholic platinic filtrate from the platinum precipitate after the removal of the alcohol and platinum, gave a solution which reduced gold chloride with avidity. It reacted with potassium ferricyanide for ferrous iron. It was therefore made alkaline with silver oxide, the precipitate filtered off, and the silver removed from both precipitate and filtrate. The solution obtained from the precipitate upon concentration gave about 2 grams of ferrous chloride, as shown by its solubility in alcohol and water and the ferricyanide reaction. The silver filtrate after removal of the silver was slightly acidified with HCl, concentrated and treated with picrolonic acid. A precipitate was obtained in the form of fine needles which after several recrystallizations came to a constant melting point at 284°C. after previous sintering at 247°C. The weight was nearly 2 grams.

0.1993 gm. substance at 23°C. and 743.8 mm. (corr.) gave 50.1 cc. N.<sup>6</sup>

0.1009 gm. substance at 23°C. and 730.0 mm. (corr.) gave 26 cc. N.<sup>6</sup>

After another recrystallization the nitrogen content did not change.

0.1514 gm. substance at 18°C. and 754 mm. gave 37.8 cc. N.

0.0947 gm. substance gave 0.0453 gm. H<sub>2</sub>O and 0.1592 gm. CO<sub>2</sub>.

0.1003 gm. substance gave 0.0473 gm. H<sub>2</sub>O and 0.1670 gm. CO<sub>2</sub>.

	Calculated for C <sub>6</sub> H <sub>14</sub> N <sub>4</sub> (C <sub>10</sub> H <sub>8</sub> N <sub>4</sub> O <sub>8</sub> ):		Found:	
		<i>a</i>	<i>b</i>	<i>c</i>
N.....	28.48	28.36	28.54	28.63
C.....	45.65	45.85	45.41	
H.....	5.58	5.38	5.27	

It would seem that a substance containing both a guanidine and an amino group, such as the calculated substance, should form a salt with two molecules of picrolonic acid. However the acidity of the solution, and the careful addition of the picrolonic acid,

<sup>6</sup> These nitrogen determinations were made by Mr. Jiklin.

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may account for the precipitation of the substance as a mono-picrolonate. In order to determine its molecular weight, an attempt was made to extract and weigh the picrolonic acid from a weighed portion after acidifying with HCl. But the hydrochloride of the base is also soluble in ether and sufficient came over with the picrolonic acid to make the result valueless. The picrolonate is very insoluble in cold water, and not readily soluble in hot water. The nitrogen and hydrogen contents place it among the substituted guanidines. A small quantity was taken up in hot water and treated with a saturated solution of picrolonic acid. A precipitate formed during the cooling. This was collected, dried and used for a nitrogen determination.

0.1169 gm. substance at 22.5°C. and 742 mm. gave 27.2 cc. N.

	Calculated for $C_8H_{12}N_4(C_{10}H_8N_4O_4)$	Found:
N.....	25.59	25.86

This substance is no doubt the dipicrolonate of the above substance. The analyses point to the mono- and dipicrolonate of either guanidine-butylamine or perhaps methylguanidine-propylamine. What the structural formula may be can be decided, definitely, only after further study.

After standing a few days the solution gave another precipitate in the form of small mounds, orange colored on the surface, light yellow inside. Some green-yellow microscopic crystals were also present. After six recrystallizations they melted at 276°C. The yield of pure substance was about 1 gram.

0.1065 gm. substance at 17.5°C. and 747 mm. gave 27.6 cc. N.

0.1454 gm. substance gave 0.0558 gm.  $H_2O$  and 0.2291 gm.  $CO_2$ .

	Calculated for $C_8H_7N_5(C_{10}H_8N_4O_4)$	Found:
N.....	29.14	29.52
C.....	42.7	42.98
H.....	4.25	4.30

This substance agrees in melting point and analyses with the picrolonate of methyl guanidine. The filtrates, remaining from the recrystallizing methyl guanidine solutions, were concentrated. From them, besides more methyl guanidine, a picrolonate was obtained in the form of yellow-green microscopic needles. These

were recrystallized, and melted at 272°C.-277°C. They were very insoluble in water. The yield was more than 0.5 gram.

0.1427 gm. substance gave 0.0672 gm. H<sub>2</sub>O and 0.2332 gm. CO<sub>2</sub>.

0.1337 gm. substance at 16.5°C. and 750.8 mm. gave 32.2 cc. N.

	Calculated for C <sub>8</sub> H <sub>8</sub> N <sub>8</sub> (C <sub>10</sub> H <sub>8</sub> N <sub>4</sub> O <sub>8</sub> ):	Found:
N.....	27.97	27.68
C.....	44.42	44.57
H.....	4.84	5.26

The analyses show it to be dimethylguanidine picrolonate and the melting point distinguishes it as being asymmetrical dimethylguanidine which melts between 275°C. and 277°C. The original solution from which the above three substances came gave another precipitate of microscopic crystals. After three recrystallizations, it came down in the form of larger red crystals. The yield was about 0.4 gram. They melted at 272°C.

0.1702 gm. substance at 17°C. and 747.5 mm. gave 45.5 cc. N.

0.1107 gm. substance gave 0.0414 gm. H<sub>2</sub>O and 0.1650 gm. CO<sub>2</sub>.

	Calculated for CN <sub>2</sub> H <sub>4</sub> (C <sub>10</sub> H <sub>8</sub> N <sub>4</sub> O <sub>8</sub> ):	Found:
N.....	30.35	30.49
C.....	40.83	40.65
H.....	4.02	4.19

This substance is doubtless guanidine picrolonate, as melting point and analyses show. The mother solution next gave about 0.2 gram of a picrolonate that decomposed in the melting tube at 119°C. It gave the reaction for neutral sulphur. A portion of the substance was allowed to stand in the sunlight for a day, after which it turned dark green. It decomposed in the air bath at 80°C. It is remarkable that this substance could have escaped decomposition from the manipulation, and reach this fraction. Finally a voluminous precipitate was obtained in the mother solution. After recrystallizing a few times it exploded in the melting tube at 348°C.

0.1094 gm. substance at 19.25°C. and 736.5 mm. gave 21.5 cc. N.

0.1023 gm. substance gave 0.0324 gm. H<sub>2</sub>O and 0.1707 gm. CO<sub>2</sub>.

After one more recrystallization the contents altered but slightly.

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0.1100 gm. substance gave 0.0391 gm.  $H_2O$  and 0.1806 gm.  $CO_2$ .

	Found.	
	a	b
N.....	21.83	
C.....	45.52	45.1
H.....	3.55	3.98

This substance is not very insoluble in water. I am not certain as to its purity. The presence of ammonium chloride is suspected since upon drying in the vacuum tube at  $120^{\circ}C$ . a small white sublimation took place forming a film on the roof of the tube. Ammonium chloride would hardly be expected to be present in this fraction. The solution always had an acid reaction, with the exception of the time of its short exposure to  $Ag_2O$ ; and ammonium chloride was previously removed quantitatively with platinum chloride. Its presence can however be explained by absorption of ammonia from the air.<sup>7</sup>

Fraction D, the alcoholic precipitate of substances insoluble in alcohol, after treatment with picrolonic acid gave a voluminous precipitate which after nine recrystallizations came down as long silky needles. They exploded at  $355^{\circ}C$ . and contained 21.38 per cent N, 44.14 per cent C and 3.5 per cent H. The substance which was very insoluble in water was not identified.

### *Histological changes.*

The histological observations are mentioned here in so far as they appear to contribute to the interpretation of the presence of the bases found. The most striking histological changes occurred in the blood, liver, kidney and brain. The blood of the vena cava and heart of all animals showed extensive ante mortem coagulation. White clots in several cases were continuous from within the heart chambers down the vena cava to its iliac bifurcation. They nearly filled the lumen of the vessel. Upon section of the liver, the vessels showed fragmented erythrocytes, many normoblasts, erythroblasts with mitotic nuclei, and a small proportion of ery-

<sup>7</sup> Seven or eight dogs were stored in the room in which this work was carried on. The atmosphere was generally ammoniacal and often very strongly so. The acid solutions during manipulation had great opportunity to absorb ammonia. Sulphuric acid was spread about for a time but there was not sufficient room for these safeguards and they had to be abandoned.



throcytes that stained brilliantly in eosin; the remaining red cells in large areas were blood shadows. Each section of the liver and lung showed a number of large mononuclear cells with eosinophile granules. There were also present a larger number of large flat cells staining very intensely in eosin. These showed no definite granulation. In places they were found to line the smaller veins like endothelial cells. In these places no endothelial cells could be observed. The cells of the hepatic cords showed advanced fatty degeneration of the protoplasm. The nuclei of large areas had disappeared entirely in places where the cell form was fairly well preserved. Such areas were surrounded by circular areas of cells in which the nuclei had become densely stained clumps of chromatin. In the livers of four of the dogs only a diffuse chromatolysis could be observed.

All kidneys showed marked congestion and hemorrhage in the cortex, some anaemic, and others, congested medullae. Some glomeruli had lost Bowman's capsule and were hemorrhagic, others were markedly congested. In some of the convoluted tubes the epithelium had degenerated.

The spleen contained a large quantity of pigment. Some of the cells showed chromatolysis.

The lung showed oedema, congestion and the blood changes mentioned.

The brain sections, which I prepared in Professor Barrett's laboratory, showed cells in the motor areas with partial loss of Nissl substance and typical tetany nuclei. Various degrees of chromatolysis were also observed in these nuclei.

The intestinal tract besides marked congestion showed in the duodenum and pyloric end of the stomach disintegrating epithelial cells. Their nuclei were converted into solid deeply staining clumps. These appeared like those in the process of extrusion from the normoblasts.

### *Symptoms.*

After the operations the dogs lived from three to five days. The wounds showed beginning healing and no infection. The postoperative period can be divided into two stages. The first stage was free from symptoms. The first portion of the second stage showed mild symptoms. The dogs were uneasy and excit-

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able, and at times appeared markedly depressed. Their pupils were sometimes unevenly dilated, and their limbs showed tremors especially after slight exertion. The last portion of the second stage was introduced by mild convulsions. The animal would lie on its side, its limbs extended and rigid. The breathing was rapid and also deep. His trunk muscles showed tremors. At times, he was oblivious to his surroundings and at other times wide awake, and appeared anxious. Such a convulsion was generally followed by a period of lassitude and fatigue, but the limbs were always more or less rigid and showed intermittently violent tremors. Some animals recovered and for a number of hours appeared quite normal. The latter part of this stage was marked by severe tetany and clonic convulsions in which the animal struggled as if to free himself. Salivation always occurred at this stage. The breathing became difficult, as from severe constriction of the air passages, and the inspirations and expirations produced high pitched and loud sounds that could easily be heard in a neighboring room. The salivation, and breathing which gradually assumed the Cheyne-Stokes type, generally was followed by death within a few hours. In some cases the bladder was distended and full of urine, in others it was constricted until the cavity was nearly obliterated.

### *Discussion.*

The occurrence of toxic bases in large quantities in the urines of parathyroidectomized dogs, observed for the first time in my work, warrants a discussion, especially since the current views regarding the function of the parathyroids make no allowance for their presence. All the urines studied contained methylguanidine. Where this substance was found in smaller quantities other guanidine bases were present, so that the excreted guanidine nitrogen approached a constant in all animals. In addition to these bases others were observed although not uniformly distributed.  $\beta$ -Imidazolylethylamine was found in three urines out of six, choline in three out of five and neurine in large amounts in one urine. In urine 5 two unidentified bases were found in the fraction where  $\beta$ -imidazolylethylamine was previously observed.

In order to determine whether any of these substances could have been split from larger molecules during analysis, the following experiments were performed.

Fifty grams of Witte's peptone were taken up in 95 per cent alcohol and the solution treated with a hot saturated alcoholic solution of potassium acetate and mercuric chloride. Some mercuric oxide formed. The mixture was hydrolyzed on the water bath under a return condenser for one-half hour, then treated with a hot saturated alcoholic solution of mercuric chloride and potassium acetate, and allowed to stand in a wide mouthed bottle for three days. A large proportion of the alcohol evaporated off. It was then placed in the cold for one day. The precipitate was filtered off and treated like that obtained from urine 5. No bases except ammonium chloride could be isolated. In order to learn if Witte's peptone would yield any bases if the hydrolysis were prolonged, the experiment was repeated, the hydrolysis lasting eight hours instead of one-half hour. Otherwise the manipulations were like those of urine 5. Besides ammonium chloride and ferrous chloride two bases were isolated from the fraction corresponding to fraction A of the urines, and another substance was found in fractions B, C, and D. There is evidence of the presence of other bases.

The picrolonate of the first substance decomposed at 130° C.

0.0965 gm. substance gave 0.0351 gm. H<sub>2</sub>O and 0.1721 gm. CO<sub>2</sub>.

0.0893 gm. substance at 22.5°C. and 734 mm. gave 20.2 cc. N.

	Calculated for C <sub>11</sub> H <sub>11</sub> N <sub>3</sub> (C <sub>10</sub> H <sub>8</sub> N <sub>2</sub> O <sub>3</sub> ):	Found:
N .....	24.34	24.76
C .....	48.53	48.64
H .....	4.04	4.07

These analyses agree well with those calculated for methyl imidazol but more data is necessary for a positive identification.

The picrolonate of the second substance softened at 192°C. and decomposed at 238°C. It was freed from picrolonic acid and converted into the gold salt; these were needles, quite insoluble in water. They melted at 232°C.

0.0261 gm. substance gave 0.0120 gm. Au.

	Calculated for C <sub>11</sub> H <sub>11</sub> N(AuCl <sub>4</sub> ):	Found:
Au .....	46.4	45.98

The substance appears to be neurine but requires further study.

The third substance became more insoluble in water with each evaporation of the alcohol during the removal of the inorganic

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salts and ammonia, so that it became sparingly soluble in water. It formed a yellow-green solution from which it crystallized in long slender yellow needles. It is soluble in hot chloroform, soluble in alcohol and ether. It gives no insoluble gold, platinum or cadmium derivatives. It gives the iso-nitrile reaction for a primary amine but no insoluble benzoyl derivative. When the aqueous solution is made alkaline with sodium hydroxide cherry red develops which is intensified by standing or more rapidly by heating. If this test solution is heated a small precipitate forms, also an oil that smells strongly of mustard oil. A solution of the substance can be heated with mercuric oxide without change, but upon rendering it alkaline and again heating the odor of mustard oil is produced and the mercury blackens. The substance is auto-oxidizable with change of the sulphur containing group. Boiling water or hot dilute acids split it into an oil, soluble in the usual organic solvents but not in water, and a substance soluble in water and the ordinary solvents from which it crystallizes in needles. The latter give a copper derivative which melts at 237°C. Sufficient substance is at hand for perhaps a proper identification and the analyses will be reported when the study is completed. The substance is of interest because of its probable genetic relation to the guanidines. The presence of a similar substance in the urines of parathyroidectomized rabbits suggested to me the search for guanidines in these urines.

The interesting investigations of Vaughan and his co-workers demonstrate that bacterial protein, egg white, and Witte's peptone after alkaline hydrolysis yield a toxic substance soluble in alcohol.<sup>8</sup> These observers found that the substance was precipitable by platinum chloride though not in crystalline form. They could isolate no bases from it<sup>9</sup> and regard it a peptone.<sup>10</sup> Although the work of these and other investigators has not yet demonstrated that the above substances can be split from Witte's peptone I can find no other reason for their occurrence than that they must have been present in combination in the original peptone. However the uniformity in composition of various samples of Witte's peptone may perhaps be questioned.

<sup>8</sup> *Zeitschr. f. Immunitätsforschung*, i, 1909.

<sup>9</sup> *Journ. Amer. Med. Assoc.*, April 22, 1905; *American Medicine*, x, p. 145

<sup>10</sup> *Trans. Amer. Assoc. Physicians*, xxvi, p. 198, 1911.

It is to be noted that the treatment given Witte's peptone in these experiments was much more vigorous than that which the urines received. The first experiment with its comparatively vigorous treatment yielded no bases as are found in these urines. In the second experiment the prolonged hydrolysis produced what are apparently neurine and methyl imidazol, and a substance which may be related to the guanidines genetically. These experiments show that prolonged alcoholic alkaline hydrolysis of a fairly large quantity of a protein produces minimal quantities of basic substances. It is therefore evident that the small quantity of protein in the urines after its comparatively mild exposure during the manipulations, could not materially alter the yields of the bases found. It is regretted that imidazol derivatives were not suspected in these urines and that the diazo reaction was neglected both before and after the removal of the proteins. The bases found may be considered to have been excreted into the urine as such nevertheless.

The histological picture of cellular disintegration may account for the excretion of these bases as products of passive protein disintegration. But something must account for the initiation of the changes. For this reason two feeding experiments were performed, none of the other dogs having been fed the day before the operation or at any time after the operation. Experiment I was performed with the dog that gave urine 1. This animal after having recovered from a violent convulsive attack and appearing quite well was fed 300 cc. of fresh milk. He drank about 200 cc. Within thirty minutes he was again in convulsions which increased in violence with exceeding rapidity and lasted two hours, proving fatal.

Experiment II was performed upon dog 5. For two days this dog had no symptoms. I then gave him some fresh sterile beef broth. He drank about 50 cc. and showed no symptoms for thirty-six hours, when they were very mild. The dog showed no stupor, but irritability. He was again fed 25 cc. of the same broth diluted, forty-eight hours after the first feeding. Within one-half hour he had symptoms of stupor with the legs in tetany and the respiration labored. The tetany was so general that the heart beat was transmitted to the abdomen in such a way that this could be seen to throb with each systole. All the muscles

## 62 Toxic Bases in Urine after Parathyroidectomy

were in severe tetany within two hours after feeding. The dog was in a stupor and had lost all volition and appreciation of his surroundings. The eyes were bulged out, and the pupils dilated. Six hours after this feeding he had passed 300 cc. of urine, and two hours after this was wide awake and panting, his jaws snapped involuntarily and rigidity of his limbs gave way to tremors. He gradually recovered, could stagger about and drink small quantities of water. This he did quite often. I left him at about one o'clock a.m. in this condition. By morning he had passed 200 cc. more urine and appeared quite well. His breathing was rapid and he appeared much fatigued and excited. By noon the tetany again set in and the convulsions increased in violence until four o'clock p.m. when he died.

These experiments show that digested proteins taken into the body have very toxic effects after parathyroidectomy. These toxic effects are due to products of intestinal and perhaps also products of parenteral digestion. Such products of digestion are normally placed in some cell molecule or stored up in some form. In the case of these animals they are free and act as toxins. In other animals where no feeding occurred the symptoms increased in violence with short intermissions until death. The violence of the symptoms doubtless followed the rate of disintegration of the body protein. This disintegration had perhaps two sources, the preparation of units to supply cells for regeneration (these could no more be used than those received from the food) and the disintegration of the famished cells. The pathological condition would thus appear to be a failure upon the part of the cells to build up their protein. This part of the metabolism of the cell is regarded as a function of the nucleus. These indications together with the formation of free nuclein elements point to a nuclein atrophy. The histological findings moreover show an active nuclein degeneration. The extensive coagulation of the blood coming from organs rich in cells and nuclei, indicates the presence of free nucleic acid in the circulation, since nucleic acid coagulates blood plasma in acid solution. The acidity of the blood is indeed indicated by the absence of iron in the erythrocytes of the blood of this region as well as by the presence of a small proportion of erythrocytes that stain intensely in eosin.

The parathyroid secretion, therefore, appears to be concerned

with anabolic processes closely related with the building of nucleins. When facilities permit these investigations will be continued.

I take pleasure in expressing my thanks to Dr. Vaughan and to Dr. Novy for facilities for carrying on this work and I am also greatly indebted to Dr. Kollig, Dr. Beyer and Dr. Huber for operating on the animals under observation.





## ON THE ACTION OF TISSUES ON HEXOSES.

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(Received for publication, May 2, 1913.)

The process of intermediate carbohydrate metabolism has been analyzed by three different methods. The first consisted in feeding the normal or diabetic individual or animal with carbohydrates and their derivatives, or substances closely related to them. The second method consisted in perfusion of normal surviving organs with the same substances, and the third was based on the action on carbohydrates of tissue extracts, or of tissues in a state of autolysis, in the presence of antiseptics. Each of the three methods has its advantages and disadvantages. The disadvantages of the first are obvious, as it does not permit of getting hold of all the intermediate products and rarely permits of a quantitative analysis of the products. The shortcomings of the perfusion method are in a lesser degree the same as of the previous, and in addition, as was pointed out recently by Embden and his co-workers,<sup>1</sup> even in experiments lasting only two or three hours the danger of bacterial growth in the circulating blood is considerable.

Finally, the third method seemed to be free of the faults of the first two, and seemed to offer the best conditions for quantitative analysis of all products formed in the reaction. For this reason, and for the reason of the comparative simplicity of the experiment, a great number of investigations have been carried out by this method. However, the greatest part of the work on glycolysis by tissues and tissue extracts suffered from either imperfect technique or from lack of critical analysis of the results of experiments. In reality, it was demonstrated that under the usual conditions, namely, in the presence of antiseptics, no actual destruction of the carbohydrate molecule takes place. Whenever there was noted in such experiments a decline in the reducing power of the sugar solution this was caused by condensation of the monosaccharide into a polysaccharide, perhaps a disaccharide.

<sup>1</sup> *Biochem. Zeitschr.*, xlv, p. 1 *et seq.*, 1912.

Up to the present date a successful dissociation of a hexose molecule outside of the body was attained only under one definite condition, namely, by the use of leucocytes in a 1 per cent Henderson phosphate solution and in absence of antiseptics. In this manner it was possible to convert hexoses into *d*-lactic acid.

This observation seemed of considerable significance since many French writers, particularly Lépine<sup>2</sup> and Mayer,<sup>3</sup> were inclined to attribute to leucocytes the function of sugar decomposition in the living organism. The experiments presented here were undertaken with the object of testing whether or not the power of converting hexoses into lactic acid was limited to the leucocytes. It was planned to subject the sugars to the action of tissues in a 1 per cent Henderson phosphate solution under perfectly aseptic conditions. It proved to be a comparatively easy matter to secure kidney tissue in a perfectly aseptic condition, but the attempts to perform similar experiments with other organs offered great difficulties, hence it was concluded for the moment to limit the experiments to kidney tissue.

The results of these experiments were identical with those in the leucocytes experiments. Through the action of the kidneys, *d*-glucose, *d*-mannose and *d*-fructose were converted into *d*-lactic acid. There was perhaps a slight difference in the quantity of sugar attacked in the two sets of experiments, the more powerful action being noted in the leucocytes experiments. The lactic acid, as in previously reported experiments, was identified as the zinc salt.

Controls with kidney tissue without the addition of the hexoses showed complete absence of lactic acid.

The fact that also by the action of kidney tissue the three hexoses yield the same *d*-lactic acid carries sufficient evidence to the effect that the mechanism of lactic acid formation is identical in all tissues.

For the bacteriological examination we are indebted to Dr. J. Bronfenbrenner.

#### EXPERIMENTAL.

*Tissues.* Rabbits were killed by exsanguination and the kidneys removed aseptically. These were reduced to small pieces and transferred to the flasks containing the sugar solution.

<sup>2</sup> *Le diabète sucré*, Paris, 1909.

<sup>3</sup> *Arch. internat. de physiol.*, ii, p. 131, 1904.

**Solutions.** The sugars were dissolved in the least quantity of water and sterilized. The 1 per cent Henderson phosphate solution was sterilized separately and then mixed with the sterile sugar solutions.

**Methods of analysis.** Sugar was estimated by reduction of Fehling's solution; the reduced copper was determined by the Volhard method.

**Lactic acid.** The solutions freed from protein were extracted with ether in a von der Heide extractor. The details of the process have been previously described. The lactic acid was determined as the zinc salt.

**Bacteriological controls.** Aerobic and anaerobic cultures were made of all mixtures prior to analysis by Dr. J. Bronfenbrenner and only those which proved free from all contamination were used.

*I. Experiments showing disappearance of sugars in mixtures of sugar and kidneys.*

*d-Glucose.*

	SOLUTION USED	NH <sub>4</sub> CNS	NH <sub>4</sub> CNS PER CC.	SUGAR	LOSS	PER CENT LOSS
	cc.	cc.	per cent			
a. At beginning of experiment.....	2	31.90	15.95	5.72		
After thirty-six hours.....	2	29.60	14.90	5.34	0.38	6.64
After hydrolysis.....	1	14.80	14.80	5.30		
b. At beginning of experiment.....	1	16.20	16.20	5.80		
After thirty-six hours.....	1	15.20	15.20	5.44	0.36	6.21
After hydrolysis.....	1	15.40	15.40	5.52		
c. At beginning of experiment.....	2	27.00	13.50	4.84		
After thirty-six hours.....	2	24.60	12.30	4.41	0.43	8.93

*d-Mannose.*

d. At beginning of experiment.....	2	27.60	13.80	4.32		
After thirty-six hours.....	2	25.00	12.50	3.85	0.27	6.28
e. At beginning of experiment.....	2	29.00	14.50	4.54		
After thirty-six hours.....	2	26.20	13.10	4.20	0.34	7.48

*d-Fructose.*

f. At beginning of experiment.....	2	31.80	15.90	5.47		
After thirty-six hours.....	2	29.60	14.80	5.05	0.42	7.68

Two kidneys from a rabbit removed aseptically and immediately taken up in 1 per cent Henderson phosphate solution, were allowed to stand at 37° for thirty-six hours, under exactly the same conditions as those to which sugar had been added. There was no measurable reduction of the Fehling's solution either before or after incubation.

## II. Experiments showing the formation of d-lactic acid.

### Glucose.

200 cc. of the glucose mixture (c, Experiment I) were extracted with ether and the lactic acid converted into the zinc salt. Yield, 0.4350 gram zinc lactate.

0.1416 gram of the recrystallized and air dried salt lost 0.0180

gram H<sub>2</sub>O on drying at 110° ..... = 12.70 per cent H<sub>2</sub>O.

Calculated..... = 12.88 per cent H<sub>2</sub>O.

0.1416 gram dissolved in 2 cc. water, total weight 2.2712 grams, gave a rotation in a 1 dm. tube of  $\alpha = -0.40^\circ$

$$[\alpha]_D^{20} = -6.4$$

### Mannose.

100 cc. of the mannose mixture (e, Experiment I) gave 0.1860 gram zinc lactate.

0.0964 gram recrystallized and air dried zinc salt lost 0.0126 gram

H<sub>2</sub>O on drying at 110° ..... = 13.08 per cent H<sub>2</sub>O.

Calculated..... = 12.88 per cent H<sub>2</sub>O.

0.0964 gram dissolved in 2 cc. water, total weight 2.069 grams, gave a rotation in a 1 dm. tube of  $\alpha = -0.30^\circ$

$$[\alpha]_D^{20} = -6.4$$

### Fructose.

100 cc. of the fructose mixture (f, Experiment I) gave 0.2083 gram zinc lactate.

0.1328 gram zinc salt lost 0.0168 gram water on drying at

110° ..... = 12.60 per cent H<sub>2</sub>O.

Calculated..... = 12.88 per cent H<sub>2</sub>O.

0.0872 gram dissolved in 1 cc. water, total weight 1.8090 grams, gave a rotation in a 0.5 dm. tube of  $\alpha = -0.16^\circ$

$$[\alpha]_D^{20} = -6.6$$

## ON CHONDROITIN SULPHURIC ACID.

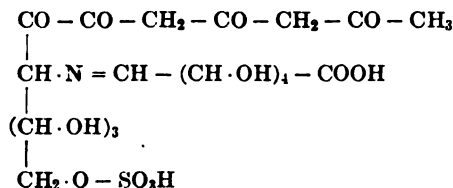
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(Received for publication, May 2, 1913.)

Chondroitin sulphuric acid has been the subject of repeated investigations, yet the information furnished by them added little to that advanced by the work of Schmiedeberg.<sup>1</sup> There is scarcely a new fact brought out by recent investigators which remained unchallenged and undisputed in the light of subsequent investigations.

According to the conception of Schmiedeberg the nucleus of chondroitin sulphuric acid is chondrosin. This, in its acetylated form (chondroitin) combines with sulphuric acid to yield chondroitin sulphuric acid.



The investigation here presented deals primarily with chondrosin. All writers are in accord in the view that chondrosin is composed of two substances in some way or other related to carbohydrates. According to Schmiedeberg the components are glucosamine and glucuronic acid. Orgler and Neuberg<sup>2</sup> contradicted Schmiedeberg on both points, claiming the components to be aminotetrahydroxycaproic acid and a hexose of undetermined configuration. S. Fränkel<sup>3</sup> further modified the view of the two preceding writers in that he interpreted the nature of the nitrogenous body as that

<sup>1</sup> Schmiedeberg: *Arch. f. exp. Path. u. Pharm.*, xxviii, p. 358, 1891.

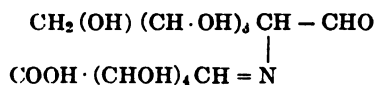
<sup>2</sup> Orgler and Neuberg: *Zeitschr. f. physiol. Chem.*, xxxvii, p. 407, 1903.

<sup>3</sup> S. Fränkel: *Ann. d. Chem.*, cceli, p. 344, 1907.

of an aminoglucuronic acid, the other component supposedly having the structure of a very labile hexose with an undetermined configuration. Still later Kondo,<sup>4</sup> working in Hofmeister's laboratory, reached the conclusion that one of the components was xylose. Finally reference has to be made to the observation of Mandel and Neuberg<sup>5</sup> that chondroitin sulphuric acid gave a test with naphtoresorcin characteristic of glucuronic acid.

It must be added here that the only crystalline substances for which absolute purity was claimed by the writer were the salts of tetrahydroxyaminocaproic acid described by Orgler and Neuberg.

It is seen from this brief review that there exists an absolute lack of agreement on the nature of the components of chondrosin. No greater is the harmony of various writers on the mode of union of the two components. According to the view of Schmiedeberg, the carbonyl group of the glucuronic acid is attached to the amino group of the glucosamine, hence giving the following expression to the molecule:



Orgler and Neuberg criticized severely the conception of Schmiedeberg as inconsistent with the properties of the substance, one of the reasons being the observation of Schmiedeberg that the reducing power of a chondrosin solution is not diminished after oxidation with nitric acid; and also on the ground of the great resistance of chondrosin against the hydrolytic action of mineral acid. Orgler and Neuberg, however, do not advance a definite view on the mode of the union of the two components. Also S. Fränkel and Kondo furnish little information in that direction. The latter however argues against the presence of a free carboxyl group in the chondrosin molecule

In course of the present investigations, which are as yet not completed, definite information was obtained of the nature of one of the components, and also of the condition of some of the characteristic groups of both components.

The difficulty of getting hold of the components is conditioned

<sup>4</sup> Kondo: *Biochem. Zeitschr.*, xxvi, p. 116, 1910.

<sup>5</sup> Mandel and Neuberg: *Biochem. Zeitschr.*, xiii, p. 148, 1908.

by the fact that chondrosin displays a great resistance towards usual hydrolytic agents, so that it was not possible to bring about hydrolysis of the molecule without simultaneously affecting the integrity of the components. This peculiarity of chondrosin was known to Schmiedeberg.

In course of the present investigation a method was found which brought about a cleavage of the chondrosin molecule permitting the isolation of at least one component. The method consisted in the use of sodium amalgam. The details of the method are described in the experimental part. The substance isolated was the usual glucuronic acid.

The substance was identified by the phenyl and parabromophenylhydrazine derivatives, by the fact that it yielded saccharic acid on oxidation with bromine, as well as by oxidation with nitric acid. The phenylhydrazine derivative had all the properties and the composition of the phenylhydrazid of the osazone. The substance was found to be identical with the one obtained under similar conditions from glucuron and first described by Thierfelder.

The *p*-bromophenylhydrazine derivative had the composition of the substance described by Guido Goldschmiedt and Ernest Zerner.<sup>6</sup> Under the same conditions we were able to obtain the identical body from pure glucuron. In accord with Goldschmiedt and Zerner we were unable to obtain the substance described by Neuberg. However, the formation of a derivative with only one molecule of hydrazine to one molecule of glucuron is *a priori* not impossible. We made no special effort in obtaining it for the reason that Goldschmiedt and Zerner's substance formed very readily from both pure glucuron and from the hydrolytic products of chondrosin.

Regarding the mode of the union between the glucuronic acid and the second component it was established first that the glucuronic acid is not bound to the amino group of the second component for the reason that the presence of an unsubstituted amino group in the chondrosin was demonstrated by the nitrous acid process.

Further, it was also made obvious that the carbonyl group of the glucuronic acid does not take part in the linking of the two components. The reasons are the following. On oxidation of chondr o-

<sup>6</sup> Goldschmiedt and Zerner: *Ber. d. deutsch. chem. Gesellsch.*, xlv, p. 113, 1913.

sin with nitric acid a product is obtained which on distillation with hydrochloric acid gives rise to a minimal quantity of furfurol, while the original substance yields a quantity required by a complex composed of one molecule of glucuronic acid and one of a carbohydrate of approximately equal molecular weight. The same oxidation product does not contain free saccharic acid, which could be identified as the acid potassium salt. However, the salt is readily obtained as soon as the oxidation product is hydrolyzed with alkali. Hence, the original oxidation product contains saccharic acid in conjugated form. This in its turn carries convincing evidence that the carbonyl group of the glucuronic acid is not the place of the union between the two components.

Whether or not the carboxyl group of the glucuronic acid is free or serves for linking the two components cannot be stated with certainty at present. Decision has to be postponed until the chemical nature of the second constituent is established. Chondrosin apparently contains only one free carboxyl group, and if the second component contains none then the conclusion will be obvious, that the carboxyl group of the glucuronic acid is present in the chondrosin molecule in a free state.

The fact that chondrosin contains both the carboxyl and the amino groups in a free state, while chondroitin sulphuric acid does not possess reducing properties, and does not react with nitrous acid to form nitrogen gas, indicates that both groups are combined in the more complex molecule with other radicals.

#### EXPERIMENTAL.

##### *Preparation of chondroitin sulphuric acid barium salt.*

Nasal septums of cattle were freed from bone and other extraneous material and ground through a meat chopper. Portions of 5 kgm. each were allowed to stand for two days with 10 liters of 2 per cent potassium hydrate solution. The extract was strained through a cloth and the residue again subjected to the same treatment with 5 liters of potassium hydrate solution and finally washed once with water. The united extracts were acidified with acetic acid and concentrated on the steam bath with an excess of barium carbonate to about half of their volume. The clear liquid was then poured off and the residue thrown on a folded filter and allowed



to drain off. This filtrate was united with the decanted liquid and the whole acidified with acetic acid and evaporated as before with barium carbonate to about 2 liters.

The separated protein and barium carbonate were removed by centrifugalization and the clear yellow liquid dropped into eight times its volume of glacial acetic acid and kept agitated by a turbine. The acid potassium salt thus obtained was filtered by suction, washed with glacial acetic acid and finally with alcohol and ether.

Two hundred grams of this product, which gave a slight biuret test, were dissolved in 10 liters of water and while the solution was kept stirred with a turbine, a solution of basic lead acetate was dropped in until complete precipitation had taken place. The lead salt, after having been washed several times by grinding in a mortar with water and filtering with suction, was suspended in 5 liters of water and with the addition of 100 grams of barium acetate and 50 cc. of acetic acid decomposed by long treatment with hydrogen sulphide with constant stirring. After standing for twelve hours the lead sulphide was filtered off and the slightly turbid solution of the barium salt precipitated by the addition of about one-third of its volume of 95 per cent alcohol. After filtering and washing with 50 per cent alcohol, then with 95 per cent, and finally with absolute alcohol and ether, the product after drying was a pure white powder, showing no trace of biuret.

This product is a mixture of the barium salts of chondroitin and chondroitin sulphuric acid. It showed no reduction of Fehling's solution and in the apparatus of Van Slyke no amino nitrogen.

0.5070 gram substance gave 7.75 cc.  $\frac{N}{10}$   $\text{NH}_3$ .

0.4650 gram substance gave 7.40 cc.  $\frac{N}{10}$   $\text{NH}_3$ .

0.4166 gram substance gave 0.1125 gram  $\text{BaSO}_4$ .

	Calculated for $\text{C}_{12}\text{H}_{17}\text{NSO}_7$ :	Found:
N.....	2.01 per cent.	(1) 2.14 per cent. (2) 2.23 per cent.
S.....	4.60 per cent.	3.72 per cent.

#### *Preparation of chondrosin.*

Fifty grams of the barium salt of chondroitin sulphuric acid were dissolved in 150 cc. of equal parts of concentrated hydrochloric acid

and water, and heated for an hour on the water bath. Barium sulphate begins to separate at once, and after one hour the solution, which is only slightly colored, shows its maximum reduction of Fehling's solution, and all the nitrogen is present as amino nitrogen. The filtered solution was evaporated in vacuum to a very thick syrup and this was taken up in about 40 cc. of hot water and poured into 500 cc. of absolute alcohol. Partial precipitation of the chondrosin hydrochloric acid salt as a nearly colorless flocculent precipitate takes place. After standing over night, two volumes of absolute ether were added and the precipitate filtered with suction and thoroughly washed with absolute ether. For a final purification the product thus obtained is dissolved in about its own weight of water and precipitated and washed again as above described. It is a quite colorless powder, which when properly washed is not hygroscopic. The yield of the first product, dried over calcium chloride for two days in vacuum, was 27 grams.

0.1966 gram substance dried to constant weight at 100° gave 12.7 cc. amino N at 21°, 764 mm. N = 3.67 per cent.

0.3319 gram chondroitin sulphuric acid barium salt gave 5.8 cc.  $\frac{N}{10}$   $\text{NH}_3$ . N = 2.44 per cent.

0.5044 gram substance hydrolyzed for one hour with one part HCl and one part  $\text{H}_2\text{O}$  gave 18.3 cc. amino N at 16°, 760 mm. N = 2.11 per cent.

#### *Cleavage of chondrosin with sodium amalgam.*

Twelve grams of chondrosin hydrochloride in 100 cc. of water were allowed to stand with 100 grams of 2.5 per cent sodium amalgam. After about twenty minutes at ordinary temperature the solution takes on a bright yellow color and at the same time an evolution of ammonia begins. The solution is then neutralized with sulphuric acid and 100 grams of sodium amalgam are again added, the temperature always being kept at about 25°. After about one hour the solution is again acidified with sulphuric acid and allowed to stand over night, after the addition of a third 100 grams of amalgam. The solution is then separated from the mercury and filtered from the sodium sulphate with the addition of some animal charcoal.

*Preparation of phenylhydrazine compound.*

The solution obtained by the above treatment was diluted to about 200 cc. and after the addition of 15 grams of phenylhydrazine in 50 per cent acetic acid allowed to stand on the water bath. After about 20–30 minutes a dark tarry material has separated together with a small amount of solid material. At this point the solution is quickly filtered with suction on a hot funnel into a hot flask and the filtrate allowed to stand for two to three hours on the water bath. After this time the solution is filled with long yellow needles to which very little of the light-colored oil adheres. The crystals were filtered and washed with warm water and then with cold absolute alcohol until no more oil drops could be discerned under the microscope. When dried in vacuum the product melts with decomposition at about 115°. Attempts to recrystallize did not effect a purification and therefore the first product was used for the analysis.

0.1188 gram substance gave 0.2484 gram CO<sub>2</sub>; 0.0634 gram H<sub>2</sub>O.

0.1278 gram substance gave 19.2 cc. N, 17°, 758 mm.

	Calculated for C <sub>14</sub> H <sub>13</sub> N <sub>3</sub> O <sub>4</sub> · 1.5H <sub>2</sub> O:	Found:
C.....	58.93 per cent.	58.80 per cent.
H.....	5.93 per cent.	6.12 per cent.
N.....	17.17 per cent.	17.33 per cent.

0.0599 gram substance in 5 cc. pyridine alcohol mixture rotated in a 0.5 dm. tube with D-light – 0.32°.

*Phenylhydrazine compound from glucuron.<sup>7</sup>*

One gram glucuron was warmed on the water bath for two hours with a little more than the required amount of normal sodium hydrate. The solution was neutralized with acetic acid, and 4 grams of phenylhydrazine in 50 per cent acetic acid and 4 grams of sodium acetate were added. After a short time crystallization of the phenylhydrazine compound in long yellow needles began and after three hours their amount had reached 1.6 grams. The material was purified by washing with cold alcohol and ether. It decomposed at about 115°.

<sup>7</sup> Thierfelder: *Zeitschr. f. physiol. Chem.*, xi, p. 395, 1887.

0.0598 gram substance in 5 cc. pyridine alcohol mixture rotated in a 0.5 dm. tube with D-light —  $0.322^{\circ}$ .

By prolonged heating in vacuum at  $100^{\circ}$  the substance loses weight but before becoming constant decomposition sets in, while at lower temperatures no loss of weight was observed.

*Parabromphenylhydrazine compound from chondrosin.*

Twenty grams of chondrosin hydrochloride were treated in the usual way with sodium amalgam, and the resulting solution, after acidifying with acetic acid, heated on the water bath with 4 grams of parabromphenylhydrazine hydrochloride. After about one hour the solution was filtered from the separated tarry material and allowed to stand three hours longer on the water bath. The impure phenylhydrazine compound obtained was washed with alcohol until the impurities had been removed and then with ether. The substance may be recrystallized by dissolving in as little as possible of a mixture of one part of 50 per cent acetic acid and one part alcohol and then precipitating by the addition of two parts of hot water.

0.0568 gram of the substance in 5 cc. pyridine alcohol mixture rotated with D-light in a 0.5 dm. tube —  $0.8^{\circ}$ .

0.0614 gram twice recrystallized under the same conditions rotated —  $0.75^{\circ}$ .

0.1454 gram substance gave 12.5 cc. N,  $22^{\circ}$ , 762 mm.

0.1126 gram substance gave 0.0118 gram AgBr.

	Calculated:	Found:
Br.....	28.95 per cent.	27.00 per cent.
N.....	10.15 per cent.	9.72 per cent.

*Parabromphenylhydrazine compound from glucuron.*

One gram of glucuron in 100 cc. of water was heated on the water bath with 2.5 grams of parabromphenylhydrazine hydrochloric acid salt, which had been purified by twice recrystallizing from dilute hydrochloric acid and washing with ether, and 2.5 grams of sodium acetate. After about one hour 0.3 gram of a yellow crystalline substance had separated. The mother liquor filtered from the first crystallization gave upon further heating 0.2 gram more of the same substance. After recrystallization from 50 per cent acetic acid and alcohol it had the following composition.

0.1436 gram substance gave 13 cc. N at 22°, 758 mm.

0.1268 gram substance gave 0.0824 gram AgBr.

0.1338 gram substance gave 0.0124 gram  $\text{Na}_2\text{SO}_4$ .

	Calculated for $\text{Br}_7\text{C}_{13}\text{H}_{17}\text{O}_5\text{N}_4\text{Na}$ ( $\text{C}_{13}\text{H}_{17}\text{N}_4\text{O}_7\text{Br}$ ):		Found:
Br.....	28.95 per cent.	20.97	27.65 per cent.
Na.....	4.17 per cent.		3.01 per cent.
N.....	10.15 per cent.	7.21	10.20 per cent.

0.0653 gram substance in 5 cc. pyridine alcohol mixture rotated in a 0.5 dm. tube with D-light — 0.90°.

*Nitric acid oxidation of the products of hydrolysis of chondrosin.*

Twenty-five grams of chondrosin hydrochloride were treated with sodium amalgam in exactly the same manner as described in the previous experiment. The solution, after having been freed from inorganic salts by precipitation with alcohol, was evaporated to a syrup. This syrup was quickly evaporated in a flat dish with nitric acid composed of one part of nitric acid, specific gravity of 1.42, and one part of water. The residue was then evaporated several times with water and finally taken up in 15 cc. of water and neutralized with potassium hydrate. Upon addition of glacial acetic acid the crystallization of the acid potassium saccharate began after a short time. After two days the yield amounted to 1.1 grams. For analysis it was recrystallized from water.

0.1253 gram substance gave 0.0427 gram  $\text{K}_2\text{SO}_4$ .

	Calculated:	Found:
K.....	15.72 per cent.	15.32 per cent.

*Nitric acid oxidation of chondrosin and subsequent hydrolysis.*

Ten grams of chondrosin hydrochloride were evaporated in a flat dish on a water bath with 10 cc. of nitric acid and 10 cc. of water. The residue was dissolved in 10 cc. of water and 5 cc. of nitric acid and again evaporated to dryness. The final residue was then dissolved in 10 cc. of water and the solution divided into two parts of 7 and 3 cc. each and neutralized in the cold with potassium hydrate. The larger portion, after addition of 2 cc. of 50 per cent potassium hydrate, was allowed to stand for two hours on the water bath and then acidified with acetic acid. After several

hours the acid potassium saccharate began to separate. The yield amounted to 0.5 gram after two days. From the smaller portion, after addition of acetic acid, only a trace of the same substance separated after long standing.

0.1276 gram substance gave 0.0440 gram  $K_2SO_4$ .

	Calculated:	Found:
K.....	15.72 per cent.	15.46 per cent.

*Brom oxidation of the products of hydrolysis of chondrosin.*

A solution of 25 grams of chondrosin hydrochloride was treated in the usual way with 2.5 per cent sodium amalgam. The solution was acidified with hydrochloric acid and allowed to stand for five days at ordinary temperature with an excess of bromine. It was then concentrated in vacuum to about 100 cc. and the principal amount of the salt separated by pouring the substance into hot absolute alcohol. The alcoholic solution was concentrated in vacuum to a syrup, taken up in water, and the halogen determined in an aliquot part. The requisite amount of lead acetate was then added to the remainder of the solution and the lead chloride and bromide removed by filtration. The excess of lead was then removed by hydrogen sulphide and the solution evaporated in vacuum to about 30 cc. It was then neutralized with potassium hydrate and after the addition of 10 cc. of glacial acetic acid allowed to stand for two days in the refrigerator. The separated crystals were filtered on suction and the product recrystallized from water. After drying it amounted to 1.6 grams.

0.1209 gram substance gave 0.0419 gram  $K_2SO_4$ .

0.1210 gram substance gave 0.0466 gram  $H_2O$ ; 0.1242 gram  $CO_2$ .

	Calculated for $C_8H_9O_8K$ :	Found:
H.....	3.65 per cent.	4.28 per cent.
C.....	27.90 per cent. <sup>a</sup>	27.98 per cent.
K.....	15.72 per cent.	15.55 per cent.

*Furfural from chondrosin after oxidation with nitric acid.*

0.4219 gram of chondrosin (calculated from the nitrogen content) was evaporated to dryness with 5 cc. concentrated nitric acid and

<sup>a</sup> Considering that one atom of carbon is contained in the ash as  $K_2CO_3$ .

5 cc. of water. After repeated evaporation with water the solution of the residue was distilled in the usual way with hydrochloric acid of specific gravity 1.06, until no more furfural was given off. Upon addition of 0.1 gram of phloroglucin 0.0076 gram of phloroglucoside was obtained, corresponding to 0.0218 gram of glucuronic acid, or about one-tenth of the amount present in chondrosin.

*Desamido chondrosin.*

Three grams of chondrosin hydrochloride in 50 cc. of water were treated with the calculated amount of silver nitrite (1.1 grams). After standing for several hours at ordinary temperature the reaction mixture was warmed on the water bath with occasional shaking. After the solution had been allowed to stand over night at ordinary temperature it was again warmed on the water bath for about two hours, after addition of 0.3 gram of silver nitrate and about 5 cc. of diluted hydrochloric acid. The excess of silver was then removed with a slight excess of hydrochloric acid and the solution evaporated in vacuum to a syrup which was taken up in very little water and poured into dry acetone. The gummy precipitate hardened quickly and was then ground with more dry acetone and washed with ether. The product was then a white amorphous powder resembling chondrosin in all its physical properties and its power to reduce Fehling's solution and gave the same amount of furfural.

0.3710 gram substance dried at 100° in vacuum gave 0.0575 gram phloroglucoside corresponding to 0.1725 gram glucuronic acid.





## ON THE SELF-DIGESTION OF THE THYMUS.

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The most abundant source of nucleic acid in the animal organism is the thymus gland. The occurrence of ferments in this gland which are capable of decomposing the nucleic acid contained therein, has been demonstrated by Kutscher<sup>1</sup> and by W. Jones.<sup>2</sup> One might naturally expect that by submitting the gland to self-digestion, all of the nucleic acid would be attacked and decomposed to a greater or less extent. In the case of autolytic enzymes occurring associated with proteins, an autolysis of the organ yields a mixture of products in which none of the original proteins can be detected. An aqueous extract of dog's liver is able to decompose thymus nucleic acid completely with the liberation of purine bases and phosphoric acid.<sup>3</sup> In certain other organs intermediate products, such as nucleosides, result as the end products of digestion.<sup>4</sup>

Regardless of the length of time (up to two months) the thymus is submitted to self-digestion, a portion of the original nucleic acid remains unattacked. The question naturally arises as to whether this portion of the nucleic acid is identical with the original nucleic acid of the gland, or whether the residue is, in reality, altered by the ferments and constitutes some intermediate product in the decomposition. By boiling with dilute sulphuric acid, the portion of the molecule of thymus nucleic acid containing the purine bases is readily attacked, and a substance resembling nucleic acids but containing only pyrimidine bases remains intact.<sup>5</sup> Furthermore, as is well known, in the hydrolysis of proteins with dilute mineral

<sup>1</sup> *Zeitschr. f. physiol. Chem.*, xxxiv, p. 114, 1901-02.

<sup>2</sup> *Ibid.*, xli, p. 101, 1904.

<sup>3</sup> Amberg and Jones: *Ibid.*, lxxiii, p. 407, 1911.

<sup>4</sup> W. Jones: *this Journal*, ix, p. 169, 1911; Levene and Medigreceanu: *Ibid.*, ix, p. 65, 1911.

<sup>5</sup> *This Journal*, xii, p. 411, 1912.

acids or proteolytic enzymes, certain groups are removed much more readily than others and a residue remains which is very resistant to the further action of the hydrolyzing agent.<sup>6</sup>

A mixture of thymus and water was allowed to digest and was filtered. The filtrate gave no precipitate of purine bases on treatment with an ammoniacal solution of silver nitrate. On the addition of acetic acid, a copious white precipitate of nucleoprotein was produced, and after filtration the purine bases could be readily detected in the filtrate by means of silver nitrate and ammonia. This explains an observation that acetic acid, added to the products of the self-digestion of the thymus, removes or decomposes some substance which interferes with the precipitation of the purine bodies. The nucleoprotein, referred to above, is evidently responsible for this, as it is well known that proteins, nucleic acids, and other substances prevent the precipitation of purine bases by means of silver nitrate and ammonia.

From the nucleoprotein thus obtained, a nucleic acid can readily be prepared. Furthermore, the residue of undissolved glandular material remaining after digestion, yields another specimen of nucleic acid. The fact that these two nucleic acids do not consist of products intermediate in the decomposition of the original material but are identical with the same, is proven by a comparison of their properties and hydrolytic products with those of the thymus nucleic acid prepared from the fresh gland.

Hence, it appears that the ferments of the thymus cannot digest all of the nucleic acid of the gland, but leave a portion completely unaltered. Whether this is due to the fact that the initial ferment which decomposes the nucleic acid is not a true catalytic agent,<sup>7</sup> to a destruction of the ferment, or to an inhibitory effect of the products of the action cannot be decided at present.

<sup>6</sup> Hammarsten-Mandel: *Textbook of Physiological Chemistry* (sixth edition), p. 128.

<sup>7</sup> The fact that certain ferments are not true catalytic agents, but that a given amount of ferment can decompose only a definite quantity of substrate and no more, has been shown in other connections: W. Jones: this *Journal*, xii, p. 34, 1912; Howell: *Amer. Journ. of Physiol.*, xxvi, p. 453, 1910; Bunzel: U. S. Dept. of Agric., Bureau of Plant Industry, Bulletin No. 238.

## EXPERIMENTAL.

Three specimens of nucleic acid were obtained and their properties compared as described below. The phosphorus was determined in the usual manner by fusion with caustic soda and potassium nitrate. The ammonium magnesium phosphate was weighed directly as described by W. Jones.<sup>8</sup> The rotation of a 1 per cent solution of each was determined in a 2 dm. tube, using Welsbach light. Since it was found that the amounts of purine bases yielded on hydrolysis depended somewhat on the conditions of hydrolysis, a specimen of each was treated in exactly the same manner. Three grams were boiled with 100 cc. of 5 per cent sulphuric acid for two hours under a return condenser. While still hot, the solution was nearly neutralized with caustic soda and made alkaline with ammonia. The guanine and adenine were precipitated as silver compounds, decomposed with hydrochloric acid, and estimated in the usual manner.

*Specimen A.* This was obtained from the fresh gland by Neumann's method.<sup>9</sup> One kilo of gland yielded 33 grams.

0.600 gram gave 0.337 gram  $\text{NH}_4\text{MgPO}_4 \cdot 6\text{H}_2\text{O}$ . A 1 per cent solution rotated  $1.25^\circ$  to the right.

*Specimen B.* One kilo of finely ground gland, 3 liters of water, and enough chloroform to prevent putrefaction were digested at  $38^\circ$  for three weeks. The material was filtered, and the filtrate treated with acetic acid. The precipitate of nucleoprotein was filtered, and dried with alcohol and ether. The nucleic acid was prepared from this nucleoprotein by heating with caustic soda in the usual manner. Four to five grams were obtained.

0.500 gram gave 0.288 gram  $\text{NH}_4\text{MgPO}_4 \cdot 6\text{H}_2\text{O}$  and with another specimen 0.400 gram gave 0.235 gram  $\text{NH}_4\text{MgPO}_4 \cdot 6\text{H}_2\text{O}$ .

A 1 per cent solution rotated in 2 dm. tube  $1.20^\circ$  to the right.

4.3 grams were heated with 25 cc. of 25 per cent sulphuric acid in an autoclave at  $130^\circ$ – $140^\circ$  for two hours. The pyrimidine bases were isolated by the usual method.<sup>10</sup> 0.225 gram thymine and 0.240 gram cytosine picrate were obtained.

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<sup>8</sup> This *Journal*, ix, p. 177, 1911.

<sup>9</sup> *Arch. f. Anat. (u. Physiol.)*, 1899, Supplement, p. 552.

<sup>10</sup> W. Jones: *Zeitschr. f. physiol. Chem.*, xxix, p. 461, 1900.

*Specimen C.* 240 grams of ground and trimmed gland, 720 cc. of water and enough chloroform to prevent putrefaction were digested at 38° for two months. The mixture was filtered and the residue on the filter treated according to Neumann's method for preparing thymus nucleic acid. 2.3 grams were obtained.

0.500 gram gave 0.308 gram  $\text{NH}_4\text{MgPO}_4 \cdot 6\text{H}_2\text{O}$ . A 1 per cent solution rotated in a 2 dm. tube 1.03° to the right.

Considering the fact that the materials were air-dried and contained probably different amounts of moisture, and further that no special attempt was made to purify the products, the following table indicates clearly the identity of the three specimens.

SPECIMEN	PHOSPHORUS PER CENT	GUANINE FROM 3 GRAMS	ADENINE PICRATE FROM 3 GRAMS	SPECIFIC ROTATION
A	7.13	0.158	0.532	+62.5°
B	7.38	0.160	0.520	+60.0°
C	7.82	0.130	0.416	+51.5°

It is, therefore, to be concluded that in the self-digestion of the thymus gland, the ferments of the gland are not capable of decomposing all of the nucleic acid within any reasonable length of time, and, moreover, the undecomposed portion of the nucleic acid appears to be identical with that prepared from the fresh gland.

## ON THE PREPARATION OF TYROSINE.

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(Received for publication, May 9, 1913.)

Owing to its great insolubility and ease of crystallization, tyrosine is one of the easiest products to isolate from a mixture of amino-acids. Its preparation from silk, however, according to the usual method is attended with considerable expenditure of time and labor and the use of large quantities of chemicals. The silk is hydrolyzed by boiling for eighteen hours with 25 per cent sulphuric acid, the mixture greatly diluted with water, and the sulphuric acid removed quantitatively with barium hydroxide. The precipitate of barium sulphate is then washed with several liters of boiling water, and the filtrate and washings (amounting to 15 liters or more) evaporated to crystallization. The product is generally highly colored, and needs to be recrystallized several times with the use of animal charcoal. A much less laborious method has been proposed by Abderhalden,<sup>1</sup> in which the hydrolysis is accomplished by fuming hydrochloric acid. The yield, however, is not as good as by the older procedure. Furthermore, raw silk is expensive as a starting material, and not always available. Considering the importance of tyrosine, especially its use in the preparation of certain synthetic polypeptides, it seemed desirable to find a method which would involve but little expenditure of time or material in its execution.

In the study of certain problems involving the self-digestion of a very concentrated pancreatic extract, it was observed that the fluid on cooling deposited beautiful white crystals of tyrosine. Since the tyrosine is very readily and quickly liberated from casein through the action of trypsin,<sup>2</sup> the use of this protein in connection with the pancreatic extract suggested itself at once. The concentrated pancreatic extract, whose preparation is described below, digests casein very readily both in the natural medium of acidity of the extract

<sup>1</sup> *Zeitschr. f. physiol. Chem.*, xlviii, p. 528, 1906; lxxvii, p. 75, 1912.

<sup>2</sup> Abderhalden and Voegtlin: *Zeitschr. f. physiol. Chem.*, liii, p. 315, 1907.

and in the mixture when made alkaline with ammonia. From such a mixture of casein and pancreatic extract, when digested at 38°, the tyrosine crystallizes, and can be readily obtained by filtration.

Finely ground and trimmed pig's pancreas is mixed with an equal weight of water and enough chloroform to prevent putrefaction, and allowed to stand at room temperature for two days. This is necessary in order that the chloroform may penetrate the gland; otherwise, putrefaction would result in such a concentrated mixture. The mixture is now placed in a thermostat and digested for twenty-four hours at 38°, cooled and filtered. The filtration proceeds quite slowly, but can be continued over night if necessary. 100–150 grams of casein<sup>3</sup> are added to each liter of the clear yellow filtrate, the mixture rendered slightly alkaline with ammonia, and digested for any convenient period from three to seven days at 38°. On removal from the thermostat, the fluid is allowed to stand over night. It is then filtered, and the precipitate well washed with cold water. The tyrosine is separated from any undigested casein or insoluble impurities by extraction of the precipitate with boiling water. Three extractions are sufficient, using 1000, 500, and 250 cc. portions of water successively. The combined extractions are evaporated to a small volume (about 250 cc.) and allowed to cool, whereupon the tyrosine separates in crusts or macroscopic crystals. For further purification the product can be recrystallized from hot water in the usual manner. One liter of the pancreatic extract and 100 grams of casein yield about 5 grams of tyrosine, while 1 liter of the extract alone yields about 1.2 grams. Analyses of three typical preparations are cited below. The specimens were not recrystallized.

- I. 0.6783 gram required 37.52 cc. 0.1N hydrochloric acid (Kjeldahl).
- II. 0.7600 gram required 42.33 cc. 0.1N hydrochloric acid.
- III. 0.8000 gram required 43.98 cc. 0.1N hydrochloric acid..

NO.	NITROGEN FOUND	THEORETICAL NITROGEN
	<i>per cent</i>	<i>per cent</i>
I	7.76	7.73
II	7.81	7.73
III	7.71	7.73

<sup>3</sup> "Casein from milk, washed," obtained from Eimer and Amend, was used in the experiments.

## A STUDY OF THE EFFECT OF CHANGES IN THE CIRCULATION OF THE LIVER ON NITRO- GEN METABOLISM.

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(Received for publication, May 17, 1913.)

### INTRODUCTION.

Hahn, Massen, Nencki and Pawlow<sup>1</sup> were among the first to undertake any very extensive metabolic experiments upon animals with the blood-supply to the liver more or less interfered with. In addition to cutting off the portal blood to the liver by means of an Eck's fistula, they ligated the hepatic artery and even went so far as to try to extirpate the liver altogether so as to totally remove its influence. The latter they accomplished only in part for they always left behind about 15 to 20 per cent of the total mass of the organ. However the partial extirpation of the liver in no way changed the results obtained by ligating the hepatic artery after an Eck's fistula; in either case the animals only survived the operation for from ten to twenty hours. It is quite evident, from their experiments, that the condition of the animal during the short lease of life left it after the operation, rendered it impossible to obtain any trustworthy information concerning what might have been the changes in metabolism had the conditions imposed by the operation permitted the animal to live.

During the course of their experiments they found that portal blood might contain, especially during protein digestion, three to five times as much ammonia as the blood of the general circulation; but after the establishment of an Eck's fistula, while they were unable to find any appreciable increase in the ammonia con-

<sup>1</sup> Hahn, Massen, Nencki, Pawlow: *Arch. f. exp. Path. u. Pharm.*, xxxii, p. 161, 1893.

tent of blood of the general circulation, the proportion of ammonia nitrogen to total nitrogen and urea in the urine was increased. They also found in their experiments, when in addition to an Eck's fistula they ligated the hepatic artery and cut away a large part (85 per cent) of the liver tissue, the ammonia not only increased in the urine, at the expense of the urea, but the ammonia content of the systemic blood actually increased, even to a point equal to that present in the portal blood, and gave rise to symptoms similar to those of ammonia poisoning, which proved fatal.

Perhaps the most interesting observation made by these investigators, as well as a most stimulating one, was, that in some of the Eck's fistula dogs, the ingestion of meat was followed sooner or later by a train of symptoms of a nervous character, similar, in some respects at least, to the symptoms of ammonia poisoning. These observations led to a number of subsequent investigations by these same authors as well as by others, viz.: Biedl and Winterberger,<sup>2</sup> Rothberger and Winterberger,<sup>3</sup> Salaskin,<sup>4</sup> Nencki and Zaleski,<sup>5</sup> and later by Macleod,<sup>6</sup> Hawk,<sup>7</sup> Fischler,<sup>8</sup> Grafe and Fischler,<sup>9</sup> all of whom dealt more or less with the relationship of ammonia to these toxic symptoms. While all agreed as to the symptoms and the increase in the ammonia content of the blood, yet all (except Salaskin) were unconvinced that ammonium carbonate, or ammonium carbamate, or in fact any other ammonia compound or  $\text{NH}_3$  compound upon which the liver is capable of acting, was the sole cause of the intoxication. Salaskin however was fully convinced that the symptoms were entirely due to ammonia.

The question to which attention was first directed in these investigations was the fate of the products of intestinal digestion and absorption which under normal conditions pass into the portal blood to be carried to the liver, before entering the general circulation. Of the nitrogen compounds formed in the intestines, am-

<sup>2</sup> Biedl and Winterberger: *Arch. f. d. ges. Physiol.*, lxxxviii, p. 140, 1902.

<sup>3</sup> Rothberger and Winterberger: *Arch. internat. de physiol.*, ii, pp. 140-141, 1905.

<sup>4</sup> Salaskin: *Zeitschr. f. physiol. Chem.*, xxv, pp. 128, 449, 1898.

<sup>5</sup> Nencki and Zaleski: *ibid.*, xxx, p. 193, 1901.

<sup>6</sup> Macleod and Haskins: *this Journal*, i, p. 319, 1905.

<sup>7</sup> Hawk: *Amer. Journ. of Physiol.*, xxi, p. 259.

<sup>8</sup> Fischler: *Deutsch. Arch. f. klin. Med.*, ciii, p. 156, 1911; *ibid.*, civ, p. 300.

<sup>9</sup> Grafe and Fischler: *ibid.*, civ, p. 319, 1911.



monia was the one to which special attention was directed, and that because it was always present, and was about the only one known to possess any very marked toxic properties. Also the action of the liver upon ammonia formed in the intestines was quite well known.

#### EXPERIMENTAL.

##### *I. Methods used for the determination of ammonia contained in the blood and their comparative accuracy.*

The methods heretofore employed for the estimation of small amounts of ammonia such as are present in the fluids of the body were not very accurate, although, in the hands of some, they led to quite accurate results as compared with the results obtained by the more modern methods. However the published results show such wide variations, only to be accounted for by the inaccuracy of the methods used, that we deemed a reinvestigation necessary. Hence it was of the utmost importance to ascertain that the method to be employed here was sufficiently accurate to come well within the limits of the small amounts of ammonia which are involved.

Hitherto the determinations of ammonia in the blood (Folin's later work excepted) were made with quantities of blood (25 to 50 grams) which at most would not contain more than 0.5 mgm. and generally not more than 0.1 to 0.2 mgm. This after being carried over into an  $\frac{N}{10}$  or  $\frac{N}{100}$   $H_2SO_4$  solution was titrated, using some one of the indicators, generally alizarin red, to determine the end-point. It is well known that most indicators do not react with the greatest precision and therefore are not applicable when very small amounts are to be taken into account. This is especially true with ammonia, which is likely to form hydrolyzed salts with the indicator and thus give an indefinite end-point. In fact when not more than 0.5 mgm. of ammonia is absorbed in 100 cc. of  $\frac{N}{10}$   $H_2SO_4$  solution, it is impossible to titrate with sufficient accuracy to even approximate the actual amount, the limits of error being several times greater than the amount of ammonia to be determined.

The method employed by us in all of the experiments was the well-known Nessler's method. This method is known to be accurate enough to distinguish 0.01 mgm. of ammonia and is therefore at least ten times more accurate than the titration methods heretofore ordinarily used. In fact it is the only known method accurate enough to determine quantities of ammonia ranging from 0.01 to 0.05 mgm.

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Preliminary controls with known amounts of ammonia showed that we were always able by this colorimetric method, to recover at least 98 per cent of the known amount of ammonia present, using amounts of not more than 0.2 mgm. in each test.

In making the estimations of ammonia in blood, we used the following procedure:

Sufficient blood was drawn from a dog's heart to make 50 grams of defibrinated blood, of which 25 cc. were put into each of two aëration cylinders (Folin), *A* and *B*. To *A* were added 5 grams of sodium chloride plus sodium carbonate sufficient to render the blood strongly alkaline, after which it was subjected to a strong current of air for six hours according to the method of Folin. To cylinder *B* was added sufficient ammonium chloride to liberate 0.5 mgm. of  $\text{NH}_3$  and then treated exactly as *A*. If cylinder *A* showed 0.35 mgm., cylinder *B* should show 0.35 mgm. plus the 0.5 mgm. added or 0.85 mgm. Unless tube *B* estimated within 10 per cent of this theoretical amount the results were rejected. However this became necessary only three or four times.

The comparative results of the colorimetric and the titration methods are shown in table I.

TABLE I.  
*Comparative results of titration and Nessler methods on normal dogs.*

NUMBER OF DOG	MGMS. OF $\text{NH}_3$ PER 100 GMS. OF BLOOD	METHOD EMPLOYED	DIET
1	1.16	Titration	Street
2	1.78	Titration	Street
3	1.17	Titration	Street
4	1.58	Titration	Street
5	1.39	Titration	Street
Average.....	1.42		
6	0.42	Nessler	Street
7	0.43	Nessler	Street
8	0.625	Nessler	Street
9	0.59	Nessler	Street
10	0.32	Nessler	Street
11	0.32	Nessler	Bread and milk
12	0.35	Nessler	Bread and milk
13	0.42	Nessler	Bread and milk
14	0.30	Nessler	Bread and milk
15	0.37	Nessler	Carbohydrate
Average.....	0.416		

The above results obtained by the titration method agree very well with those obtained by other workers, by similar methods, but represent about three to four times the actual amount of ammonia present, as shown by the Nessler method. However it must be stated that certain other investigators, especially Horodynski, Salaskin and Zaleski<sup>10</sup> obtained results agreeing very closely with the above (Nessler's method). Their tables show an average ammonia content for arterial blood (dogs) of 0.41 mgm. per 100 grams; for portal blood (fasting), 1.29 mgm., and for portal blood during protein digestion of 1.85 mgm.

Folin,<sup>11</sup> working with cat's blood, employing the calorimetric method and using small quantities of blood drawn into weighing bottles containing sufficient potassium oxalate to prevent coagulation, recovered extraordinary small amounts of ammonia, only traces in the arterial blood and about 0.15 mgm. per 100 grams in the portal blood. He aerated the blood for a short time, not more than thirty minutes, thinking that a longer period might give rise to decomposition ammonia. With dog's blood we were unable to confirm his findings, the method given for arterial blood 0.35 mgm. per 100 grams and for portal blood (same dog during digestion), 0.68 mgm.

TABLE II.

*Comparison of the ammonia content of the portal blood with that of the common carotid of the normal dog.*

DOG NO.	KIND OF BLOOD	MGM. OF NH <sub>3</sub> PER 100 CC. OF BLOOD	METHOD
I	{ Portal.....	0.800	Nessler
	{ Arterial.....	0.420	Nessler
II	{ Portal.....	0.860	Nessler
	{ Arterial.....	0.430	Nessler
III	{ Portal.....	1.220	Nessler
	{ Arterial.....	0.625	Nessler

These dogs were examined at the height of protein digestion, about five hours after feeding 1.5 pounds of lean meat each. The results agree in general with those of other investigators and leave

<sup>10</sup> Horodynski, S. Salaskin and J. Zaleski: *Zeitschr. f. physiol. Chem.*, xxxv, p. 246, 1902.

<sup>11</sup> Folin and Denis: *this Journal*, xi, p. 161, 1912.

no doubt but that ordinarily the portal blood carries about twice as much ammonia as the systemic blood. Although not shown in the above tables our analyses were confirmatory of the findings of previous workers, that the blood of the hepatic veins contains no more ammonia than is present in the arterial blood, thus proving that the excess ammonia contained in the portal blood is taken out during its passage through the liver.

Repeated examinations of the systemic blood of individual dogs showed that the ammonia content was practically constant for each animal examined, irrespective of the diet, state of digestion or condition of the bowel. While repeated examinations of the portal blood of the same dog are not permissible, dogs examined under different conditions showed a variation in the ammonia content of the portal blood. Blood taken during high protein digestion or when the bowels were full, especially when much fecal matter was present, always gave a high ammonia content as compared with fasting animals, or after the lower bowel had been cleansed by purgation. Just what proportion of the ammonia formed in the intestines as the result of protein digestion is difficult to estimate; at least a good percentage of it is given off from the feces in the lower bowel (Folin).<sup>12</sup>

## II. Eck's fistula dogs.

Out of a total of thirty-five dogs with Eck's fistula, only three responded to meat feeding with the typical symptoms described in the literature. One of these we wish to speak of more or less in detail.

Dog I. This dog, a large female weighing 12 kgm., was killed when in good health fifteen months after an Eck's fistula operation and had suffered seven attacks of meatintoxication. Following the operation, she was kept on a bread and milk diet for thirty days, during which period she lost some flesh, but otherwise was apparently normal. She was then put on a generous allowance of raw lean beef which she ate ravenously, seeming to enjoy the change. She gradually became lively and restless and in about ten days was showing symptoms, simulating the restlessness and irritability so characteristic in mild cocaine intoxication. These

<sup>12</sup> *Loc. cit.*

symptoms increased up to the fifteenth day of the meat feeding when the dog became delirious and during the following day suffered frequent attacks of clonic convulsions. All food was refused, but warm milk was administered through a stomach-tube. After the first milk feeding, recovery began to take place and the animal was normal in forty-eight hours.

The symptoms, as they developed, suggested a gradually increasing cerebral stimulation, beginning first with the highest areas (caffein-like), followed by a like action upon the motor areas (cocaine-like), and passing on to the medulla, so that

TABLE III.

*Ammonia in the blood of Dogs 1 and 25, before operation; after operation; periods of good health; and periods of meat intoxication.*

*Dog 1.*

DATE	NH <sub>3</sub> IN MGM. PER 100 cc. BLOOD	DIET	REMARKS
1910			
Feb. 10	0.4	Mixed	Eck's fistula performed.
July 14	0.42	Bread and milk, 15 days	Dog in good condition.
July 20	0.52	Meat, 5 days	Blood drawn at height of digestion.
July 26	0.6	Meat, 11 days	Dog. very irritable, active, in delirium.
July 27	0.52	Eaten nothing for 36 hours.	Clonic convulsions.
Aug. 2	0.49	Bread and milk, 6 days	Dog in good condition.
Aug. 11	0.53	Meat and Liebig's ext. (2 oz. per day) for 5 days.	No toxic symptoms.
Aug. 17	0.66	Meat and Liebig's ext., 15 days	Typical toxic symptoms; blood-pressure low, and blood dark-colored.
Nov. 14	0.49	Bread and milk, 30 days	Dog in good condition.
Nov. 18	0.48	Bread and milk, 30 days	Dog in good condition.
1911			
May 12	0.43	Mixed	Killed.

*Autopsy.* Abdominal viscera free from adhesions or other evidences of collateral circulation. The liver was small and had undergone fatty necrosis almost to complete destruction. Kidneys normal. Ratio of ammonia nitrogen to urea nitrogen, 1:32.

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TABLE III—Continued  
Dog 25.

DATE	NH <sub>3</sub> IN MGM. PER 100 CC. BLOOD	DIET	REMARKS
1911			
Apr. 29	0.215	No food for 24 hours	Just before Eck's fistula operation. 9 a.m.
May 1	0.376	Cooked meat	Operation at 10.30. Blood drawn 1 hr. after meat feeding.
May 9	0.54	Cooked meat (not eaten)	Dog had eaten nothing for 12 hours; very weak, although no toxic symptoms present. 9.30 a.m.
May 9	0.502	Meat (not eaten)	Marked toxic symptoms; convulsions—clonic upon tonic. 10.30 a.m.
May 23	0.38	Mixed diet since May 9	Dog in good condition.
June 2	0.56	Meat diet from May 23	Dog in hypersensitive condition, bordering on convulsions.
June 12	0.5	Mixed diet	Dog improved but still noisy.
June 14			Killed.

*Autopsy.* Abdominal cavity perfectly clear. Fistula was wide open. No adhesions.

the final stage gave a very mixed set of symptoms, hard to define pharmacologically.

It is worthy to note that it required about twelve to fifteen days of intensive meat feeding to bring on the intoxication. An ordinary mixed diet was not sufficient. As stated this dog was subjected to intensive meat feeding seven times, and was just as susceptible to the intoxication fifteen months after the operation as one month—no tolerance nor immunity having been established.

There was no appreciable increase in the ammonia content of the blood following the fistula operation, it being about 0.4 mgm. per 100 grams before and after the operation; but the proportion of the ammonia nitrogen to the total nitrogen and urea in the urine was increased, almost wholly at the expense of the urea. This disarrangement of the nitrogen constituents of the urine almost wholly disappeared after eight to ten months. As shown in table III, the ammonia content of the blood was somewhat greater

during the periods of meat feeding, especially at the beginning of the acute symptoms, as was also the ammonia in the urine. During the acute intoxication the urine was diminished not more than 50 to 75 cc. per 24 hours as compared with 250 to 300 cc. when on a mixed non-toxic diet.

As a further study of the toxic action of the portal blood when saturated with the products of protein digestion, three dogs were operated on five hours after the ingestion of 2 pounds each of Hamburger steak. These animals only partially recovered from the effects of the operation. In two or three hours they were walking about, but gradually lapsed into a comatose condition and died in from ten to twenty-four hours. At autopsy, the fistulae were found wide open and no congestion or stasis of the portal circulation was present.

To compare with these, dogs were operated on three hours after the ingestion of 300 cc. of milk, containing 50 grams of starch, 50 grams of glucose and 30 cc. of olive oil. These dogs recovered as promptly as did the dogs which were operated on after one day of fasting. This is taken as an indication that when the portal blood, taken at the full tide of protein digestion, is suddenly turned into the general circulation, it may produce profound depression, coma and death; whereas if time is given for the adaptation of the body to the Eck-fistula before the meat feeding, as was usually done, there was no such profound depression. However, other experiments showed that it took only a short time for the animals to become adapted to the new condition. Several dogs were operated on after having fasted for forty-eight hours and meat feeding was instituted twenty-four hours afterwards. These dogs seemed no more susceptible to meat poisoning than when the meat feeding was instituted thirty days after the operation. In fact, none of these dogs proved susceptible.

### *III. The effects of Eck's fistula and ligation of the hepatic artery.*

1. *Eck's fistula and hepatic artery ligated at the same time.* Dogs so operated upon recovered from the immediate effects of the operation but as a rule, after from eight to ten hours, they began to lapse into a comatose condition and died within twenty-four hours. Some of the animals did not live for more than twelve hours, in

fact these dogs behaved very much like those on which the Eck's fistula was made at the height of protein digestion. Autopsies made immediately following death showed the liver undergoing rapid autolysis and, in those cases where the animal lived for twenty-four hours, the whole organ was in a state of disintegration emitting a fatty acid odor. In fact, some gas was free in the abdominal cavity and generally from 25 to 50 cc. of dark-colored fluid. Blood drawn during the coma showed a marked increase in the ammonia content which was equal to that found in blood immediately following death.

2. *Hepatic artery ligated several weeks after Eck's fistula operation.* In four dogs so operated upon a somewhat different state of affairs resulted as shown in table IV. One of these (No. 14) had the hepatic artery ligated on April 18 and lived until the morning of April 21, when life was terminated on account of an accident. This animal ate meat and passed about 300 cc. of urine. At death 25 cc. of urine were taken from the bladder which contained 4.5 per cent total nitrogen of which about 60 per cent was urea and 20 per cent ammonia. Autopsy showed: no adhesion, the hepatic artery ligated, the fistula open 1 cm. in diameter, and the portal vein completely closed. The liver was small and firm with a few darkened areas scattered over the surface. Had not life been terminated by accident, everything indicated that the animal would have lived for several days longer.

The other three dogs died within thirty hours, one only lived for nine hours and died while in a state of strychnine-like convulsions. The other two (23 and 25) which lived for twenty-four hours or more, recovered from the immediate effects of the operation, ate meat and drank water. About fifteen hours after the operation, they began to show signs of hypersensitiveness which developed into convulsions, both clonic and tonic in character, in which condition they died.

The autopsies showed: no peritonitis, no fluid in the abdominal cavity, the hepatic artery and the portal vein completely shut off, and the fistulae were open and about 1 cm. in diameter. The livers were well preserved, not having undergone any marked acute autolysis. Adhesions were quite extensive especially in the lower surface of the liver, apparently sufficient to furnish a fair supply of blood to the organ.



These dogs, although their livers showed no acute autolytic changes as did the livers of the dogs upon which both operations had been made at the same time, lived, with the exception of dog 14, only a few hours longer than the latter. Only a few cubic centimeters of urine were excreted, highly acid, and as shown in table IV the ammonia content of the blood had increased to a point apparently equal to that necessary to cause poisoning.

#### *IV. Dogs showing digestive disturbances.*

Of the remaining dogs, ten ran rather a peculiar course. They recovered readily from the immediate effects of the operation, ate well of any food for which dogs have a liking, but in a few days began to lose weight and ran down in general health. They suffered more or less from diarrhoea, the feces being clay-colored with a fetid odor and contained an excess of fat. It was almost impossible to keep these animals even in a fair state of nutrition. Most of them went from bad to worse and died in from six to eight weeks in an extreme state of inanition. Autopsies on these animals showed the fistulae open, with practically no adhesions. The course run by these dogs was quite similar to that after the establishment of a permanent biliary fistula in which case the normal nutrition is hard to maintain. Also if the pylorus be closed and a gastro-enterostomy be made 40 cm. distal to the pylorus, dogs will suffer like digestive disturbances and as a rule die within two months from inanition. While quite foreign to the subject in hand, it might be stated in this connection that in some dogs, partial removal of the pituitary gland is followed by a like train of symptoms and death in about the same length of time. In seven of these dogs we could not make out any increased ammonia content of the blood. In three, however, the ammonia content of the blood was distinctly increased as shown by the following figures.

Before operation (mixed diet): 0.35, 0.42, 0.39 mgm. per 100 grams.

After operation (average of three estimations): 0.51, 0.75, 0.65 mgm. per 100 grams. The urine showed the same metabolic changes as occur in starving animals.

These figures are almost as high as those given in table II for the portal blood, and are as high as obtained from other dogs when in convulsions from meat intoxication.

## 98 Nitrogen Metabolism after Eck's Fistula

TABLE IV.

*Ammonia in the heart's blood of four dogs with Eck's fistula after subsequent ligation of the hepatic artery.*

*Dog 14.*

DATE	NH <sub>3</sub> IN MGM. PER 100 CC. BLOOD	DIET	REMARKS
1912			
Jan. 28	0.4	Fasting 24 hours	9 a.m.—10.30, operation.
Feb. 9	0.52	Meat + 32 gms. Liebig meat ext. per day since Jan. 30	Marked meat poisoning. 10 cc. of 10 per cent calcium lactate injected intraven- ously, after which dog be- came quiet and slept several hours. No more convul- sions, recovery complete in 48 hours.
Feb. 14	0.49	Cooked meat and milk	Dog in good condition.
Mar. 25	0.512	Meat + 64 gms. Liebig meat ext.	Dog very irritable, ill natured and very noisy.
Apr. 14	0.52	Same diet	Very noisy, continued rest- lessness but no convulsions.
Apr. 18			Hepatic artery ligated.
Apr. 19 a)	0.761	Cooked meat	At ten, blood was drawn and two estimations made.
Apr. 19 b)	0.747		Dog in good condition.
Apr. 20			Abdominal wound broke open
Apr. 21			and dog had to be killed while seemingly in good condition.

*Autopsy.* No infection; liver small and blackened in spots; hepatic artery ligated; no adhesions; fistula 1.5 cm. in length.

*Dog 23.*

1911			
Apr. 25	0.32	Fasting	Blood drawn 9 a.m. Eck's fis- tula performed at 10.30.
Apr. 28	0.40	Bread and milk	Dog in good condition.
May 16			Hepatic artery ligated at 10 a.m.
May 17	0.92	Bread and milk	10 a.m. Dog in good condi- tion.
May 17	2.47	Bread and milk	Dog in convulsions (4. p.m.) 4.45, died.

*Autopsy.* Hepatic artery ligated; liver small and undergoing rapid autolysis; fatty acid odor.

TABLE IV—Continued.

*Dog 25.*

DATE	NH <sub>3</sub> IN MGM. PER 100 CC. BLOOD	DIET	REMARKS
1911 June 12	0.5	Mixed diet	Dog recovering from meat poisoning, hepatic artery ligated (Eck's fistula Apr. 29).
June 13	0.83	Milk (little taken)	Dog in good condition.
June 13 5 p.m.	0.96		Symptoms of cerebral excitations.
June 13 10 p.m.			Dog died in convulsions.

*Dog 30.*

Apr. 25	0.39	Fasting	Eck's fistula made.
June 2	0.55	Meat diet	Dog hypersensitive, very restless and noisy.
June 9	0.54	Meat diet	Hepatic artery ligated at 10 a.m.
June 9 5 p.m.	1.15		Convulsions.
June 9 7 p.m.			Died in opisthotonos.

*V. Dogs—normal after operation.*

The remaining dogs (22) showed neither the symptoms of meat poisoning nor the digestive disturbances of the group just described. They recovered quickly from the effects of the operation, ate heartily of meat, bread and milk or any other sort of food offered them. For a time they were lively, apparently hypersensitive, but in a few weeks settled down to a normal condition. As a rule they gained in weight. Autopsies showed very marked adhesions of the liver to the surrounding viscera with more or less stenosis of the fistula. In most cases, the liver was firmly adherent to the pancreas, duodenum, and to other parts of the intestine with which it came in contact. The adhesions were very vascular and appeared to be sufficient to take the place of the portal vein. In those

cases where the adhesions were most extensive, the stenosis of the fistula was almost complete. In one case the fistula was completely closed and even the main trunk of the portal vein was obliterated. Yet the animal lived and enjoyed good health.

The significance of such adhesion as a protection against meat poisoning and digestive disturbances has been pointed out by Rothberger and Winterberger.<sup>13</sup> Under such conditions the liver apparently functions in the normal way, the blood going to the liver through the adhesions instead of the portal vein.

These findings corroborate the earlier findings of the St. Petersburg school already cited, namely, that an Eck's fistula does not necessarily cause an increase of ammonia in the systemic blood, but that in fistula-dogs susceptible to meat intoxication, ammonia does accumulate in the blood of the general circulation considerably above that normally present, and that after the ligation of the hepatic artery upon an Eck's fistula, the ammonia in the systemic blood may increase to an amount equal to or greater than that normally present in the portal blood, and that animals subjected to such operations soon die showing symptoms such as occur in acute ammonia poisoning.

#### DISCUSSION.

The change in the nitrogen metabolism most apparent under the conditions imposed by an Eck's fistula is that the proportion of ammonia nitrogen to urea nitrogen in the urine is increased without materially changing the total nitrogen output. Also some investigators have reported a temporary increase in the uric acid and lactic acid outputs, the latter being more lasting than the former. This suggests two possibilities; either the liver cannot act upon the nitrogen compounds contained in the portal blood, which under normal conditions it changes to urea, when they are carried to it in the arterial blood, or that these same nitrogen compounds are excreted so rapidly by the kidneys that only a minimum ever reach the liver.

Evidence, largely indirect, is not wanting that the former is the correct explanation. After an Eck-fistula, the liver ceases to store up glycogen in the usual quantities. The liver of such animals, even when well fed upon carbohydrate, corresponds in glycogen

<sup>13</sup> Rothberger and Winterberger: *loc. cit.*

content to the liver of fasting animals, while the muscles may contain quantities of glycogen corresponding to those found in well-fed animals (De Filippi).<sup>14</sup> In other words, the liver ceases to participate in the formation of glycogen, although, unlike the ammonium compounds, the sugar is not excreted in the urine but evidently circulates sufficiently long in the blood to come intimately in contact with the liver tissue as such contact takes place with the arterial blood. An Eck's fistula seems to influence very little the ability of an animal to utilize sugar, and it can hardly be said to lessen the sugar tolerance at all.

The effect of an Eck's fistula upon the formation of bile, might be cited as additional evidence that the liver does not act upon the precursors of bile when deprived of the portal blood. As showed by Voegtlin and Bernheim<sup>15</sup> and corroborated by us, ligation of the common bile duct after an Eck's fistula induces no jaundice nor any accumulation of bile in the blood. This observation suggests one of two possibilities; either the liver forms bile from the constituents of the portal blood only, or the portal blood contains some substance which stimulates the liver to bile formation. This is contrary to the generally accepted view that the liver forms bile from the blood irrespective of its source.

*Have we any evidence that a like condition obtains in regard to the formation of urea by the liver?* It may be taken as proven that the quantities of ammonia in the portal blood above those present in the systemic blood are converted into urea by the liver and are not permitted to enter the general circulation at all; and that they constitute at least one of the sources of urea. It is also quite evident that when the portal-blood ammonia is diverted into the general circulation, and permitted to come in contact with the liver tissue by way of the hepatic artery only, it no longer serves as a source of urea but is eliminated in the urine as such. When we take into consideration that the kidney epithelium is very readily permeable to ammonia compounds in general, the above argument loses some of its force, for the kidneys, coming, as they would, in competition with the liver in ridding the blood of any excess of ammonia, might place the liver at a very great disadvantage.

In our series of experiments, dog 14 (table IV) furnished some information on this question. As already stated this dog, which

<sup>14</sup> F. de Filippi: *Zeitschr. f. Biol.*, xlix, p. 511, 1907; l, p. 38, 1908.

<sup>15</sup> Voegtlin and Bernheim: *Journ. of Pharm. and Exp. Ther.*, ii, p. 455, 1911.

was susceptible to meat intoxication, had the hepatic artery ligated several weeks after the Eck's fistula was established and lived in good condition for forty-eight hours afterwards with every indication that life would have continued longer, had it not been terminated by accident. The animal drank water and ate meat during the short lease of life after the last operation and passed 300 cc. of urine. At death the stomach contained food, and the urine (25 cc.) which was taken out of the bladder when killed, contained 4.5 per cent total nitrogen of which about 60 per cent was urea and 20 per cent ammonia nitrogen. We could find no evidence that, after the ligation of the hepatic artery the liver had received any blood by way of the portal vein or hepatic artery, both being completely closed. This, while it does not bring forward any conclusive evidence that the liver does not form urea from urea precursors when they are presented to it in the arterial blood, does furnish evidence, at least, that urea may be formed by tissues other than those of the liver. But the fact that the ligation of the hepatic artery did not materially add to the disarrangement of the nitrogen constituents in the urine occasioned by the Eck's fistula, argues that the liver does not form urea from ammonium salts when circulating in the systemic blood alone.

This together with the evidence furnished by dog 1 (table I), which lived fifteen months with an Eck's fistula, was subjected to meat intoxication seven times, and the liver of which had undergone degeneration almost to complete destruction, yet excreted, during the last two months of life, urea up to 80 per cent of the total nitrogen, while for some weeks following the operation the urea nitrogen was only 60-65 per cent of the total nitrogen excreted, suggested the following speculation: The liver not only does not form urea from ammonia or ammonium compounds when presented to it in the arterial blood, but that, when the liver ceases to be a factor in the formation of urea, the other tissues of the body gradually assume the work in this regard, normally done by the liver. This is paramount to saying; that no specific tissue of the body enjoys the sole monopoly to form urea in the process of nitrogen metabolism, but that urea is formed by the tissues in general, at least primarily; and that after the removal of certain specialized tissues (organs) which possess this power to a greater degree than the general tissue, there follows a reversion to the original form of metabolism by the tissues in general which may be sufficient to

maintain the nutrition of the body without the intervention of specialized tissue.

While these changes in metabolism occur after an Eck-fistula, they are not wholly characteristic of the conditions of life imposed by the operation. Ligation of the hepatic artery, alone, brings about the same changes as does an Eck's fistula, *i.e.*, the ammonia compounds in the portal blood, which normally are changed into urea by the liver, are no longer changed but pass into the general circulation to be excreted in the urine as such (Doyon and Dufourt).<sup>16</sup> Phosphorus poisoning, with its characteristic changes in the liver cells, brings about a similar condition in metabolism, *i.e.*, ammonia is not changed to urea and oxidizable acids may appear in the urine.

It is well known that after an Eck's fistula the liver soon begins to undergo fatty necrosis which eventually invades the whole organ. This change in the nutrition of the organ may be responsible, in large measure, for the changes in metabolism so noticeable after an Eck's fistula. If so, then the fact that the liver does not perform its chemical work when shunted out of the portal circulation would have no bearing upon the question, whether or not the liver acts chemically upon substances circulating in the systemic blood only.

Ligation of the hepatic artery is followed by no such nutritive changes in the liver, therefore the suspension of chemical work occasioned by ligation of the artery must be due to a diminution in the oxygen supply as suggested by Doyon and Dufourt already cited.

From what has been said, it seems that the exclusion from the liver, by way of the portal vein, of the products of protein digestion and absorption as they appear in the portal blood and the excretion of at least some of the precursors of urea (ammonia) in the urine is not incompatible with life and good health. Even when long continued, the augmented excretion of ammonia in the urine is without injurious effects on the kidneys (dog 1).

However, fistula dogs are susceptible to meat intoxication, while animals with the hepatic artery ligated are not. This then might be taken as evidence that the ammonium compounds absorbed

<sup>16</sup> Doyon and Dufourt: *Compt. rend. de l'Acad. des Sci.*, 1898, p. 419; *Arch. de physiol. norm. et path.*, x, pp. 522-37, 1898.

from the intestines, and which are convertible to urea by the liver, are not the toxic agents in meat intoxication.

While we have attempted to reduce to a minimum the influence of the liver on protein and carbohydrate metabolism, we cannot regard it as unimportant in certain other respects. It evidently has the power to destroy certain substances formed from proteins during intestinal digestion and absorption, which are poisonous when not allowed to enter the portal circulation. This might be taken as further proof that the liver acts upon substances only when circulated through it in the portal blood. Again, the liver cannot be shunted out of the portal circulation without, in at least a good percentage of cases, affecting the digestion. As already stated, ten of our dogs died of inanition in from four to six weeks after the operation. The symptoms exhibited were quite similar to those following the establishment of a permanent biliary fistula, and even more so to the symptoms occurring after the pylorus is closed and a long looped (40-45 cm.) gastro-enterostomy made. In the latter case, dogs always die from inanition in from five to eight weeks, presumably from some interference with the formation of the digestive juices in the duodenum, probably due to the lack of the formation of "secretin." It might be imagined that an Eck's fistula, interfering as it does with the formation of bile, would bring about a condition similar to that following a permanent biliary fistula, or we might conceive, as has been suggested by others, that the liver forms some substance of the nature of an internal secretion, the formation of which is interfered with by an Eck's fistula. That the former will not suffice to explain the occurrence of digestive disturbances is shown by the following observation: Dogs having both an Eck's fistula and the common bile duct ligated may and often do return to a normal state of health, especially if extensive adhesions form about the liver, although no bile is permitted to enter the duodenum. This is rather paradoxical. After the formation of adhesions, we would expect the resumption of the formation of bile due to the fact that much of the return flow of blood from the intestines now circulates through the liver. There seems to be no resumption in the formation of bile, neither are there any digestive disturbances. This indicates that the adhesions bring about a partial return flow of portal blood which stimulates the liver to form some substance which offsets the deleterious effects occurring when no adhesions are formed.



## ON FAT ABSORPTION.

### II. ABSORPTION OF FAT-LIKE SUBSTANCES OTHER THAN FATS.

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The manner in which fats are absorbed is still largely a matter of opinion rather than of proven fact. Aside from the possibility of absorption in other channels than the chyle, it is a question whether saponification is a necessary preliminary to absorption, or whether some of the fat may be carried into the chyle intact either as an emulsion or in some sort of solution. It is generally recognized that under ordinary conditions of feeding most if not all of the fat which enters the blood through the chyle has been broken down and rebuilt by the intestine, but there is much evidence to show that under other conditions, especially in the feeding of large amounts of fat, food fats may be transferred to the fat depots of the animal body without marked change.

Dyes which are soluble in fats and fatty acids but not in aqueous soap solutions, have been shown to be absorbed in quantity.<sup>1</sup> Even bacteria have been shown to be aided in their passage through the intestinal wall by fats.<sup>2</sup> It is reasonable therefore to suppose that any substance which is soluble in fats and fatty acids might similarly be carried through the intestinal wall and that therefore at times fats may be absorbed in this way.

It is not possible to test this point with fats themselves, since fat so transported could not be distinguished from the synthesized fat of the chyle. Three classes of substances which have been used for this purpose are: (a) the readily saponifiable esters of the fatty acids other than triglycerides, (b) difficultly saponi-

<sup>1</sup> Mendel and Daniels: this *Journal*, xiii, p. 71, 1912.

<sup>2</sup> Ravenel: *Journ. of Med. Res.*, x, p. 460.

fiable esters of the fatty acids (cholesterin, etc., esters of wool-fat), and (c) the petroleum hydrocarbons.

*The readily saponifiable fatty acid esters.* Munk and Rosenstein,<sup>3</sup> working with a case of human chyle fistula and also with dogs, fed cetyl palmitate and amyl oleate and demonstrated that none of these substances appeared in the chyle fat. Frank,<sup>4</sup> after feeding dogs with ethyl esters of various fatty acids, found in the chyle the triglycerides of the fatty acids, but no trace of alcohol or its esters. Bloor<sup>5</sup> fed to dogs highly optically active iso-mannid esters of the fatty acids and could find no optically active fatty substance in the chyle. Frank and Argyris,<sup>6</sup> in similar experiments with the monoglycerides of the fatty acids derived from lard, found that the chyle fat contained only triglycerides. The saponifiable esters of the fatty acids are thus well absorbed and, as none of the unaltered esters appeared in the chyle, saponification would appear to be a necessary preliminary to absorption; although the reason for their absence from the chyle might also be that they merely did not escape the excellent facilities for hydrolysis in the digestive tract.

*Unsaponifiable fatty acid esters.* The cholesterin and iso-cholesterin esters which are the main constituents of wool-fat are very resistant to lipases, and since they form extremely fine emulsions with water, they would seem to be very suitable substances for testing the absorption of fat-like substances in other than water-soluble form. The feeding experiments of Connstein<sup>7</sup> with these substances indicate, however, that they are not absorbed.

*The petroleum hydrocarbons.* The petroleum hydrocarbons are soluble in fat and fatty acids and when so dissolved, emulsify well with dilute alkalies, but cannot be reduced to water-soluble form in the intestine. In a series of experiments on rats with an emulsifiable mixture of one of these substances (vaseline) and lard, Henriques and Hansen<sup>8</sup> found that the hydrocarbon could be quantitatively recovered from the feces and concluded that

<sup>3</sup> Munk and Rosenstein: *Virchow's Archiv*, cxxiii, pp. 230, 484.

<sup>4</sup> Frank: *Zeitschr. f. Biol.*, xxxvi, p. 568.

<sup>5</sup> Bloor: this *Journal*, xi, p. 429, 1912.

<sup>6</sup> Argyris and Frank: *Zeitschr. f. Biol.*, lix, p. 143.

<sup>7</sup> Connstein: *Arch. f. (Anat. u.) Physiol.*, 1899, p. 30.

<sup>8</sup> Henriques and Hansen: *Centralbl. f. Physiol.*, xiv, p. 313, 1900.

there could be no absorption of fat in other than water-soluble form. Bradley,<sup>9</sup> after feeding an *emulsified* mixture of another hydrocarbon (liquid albolene) and olive oil, found that the chyle fat consisted of about equal parts of the two substances, and came to the opposite conclusion—that fats may be absorbed as emulsions.

The results of the work, cited above, as a whole, go to show that only those substances were absorbed which could be reduced to water-soluble form. In view of the contradictory nature of the evidence on the petroleum hydrocarbons and the meagre data with respect to the unsaponifiable esters it seemed desirable to make a more detailed examination regarding the absorption of these two classes of substances.

#### I. THE ABSORPTION OF HYDROCARBON OILS.

The hydrocarbon oils used were those sold under the trade names of "liquid albolene" and "white vaseline." They were fed alone and in emulsified and unemulsified solutions in olive oil or cocoanut oil.

##### *A. Feeding experiments with unemulsified (but emulsifiable) mixtures of the petroleum hydrocarbons.*

First, feeding experiments were conducted in which solutions of the unemulsified hydrocarbon oils were fed to cats and the amount of unabsorbed oils determined by extraction of the feces. For this purpose they were dissolved in equal parts by weight of a fatty oil and fed together with a sufficient ration of boiled lean meat freed from visible fat. Olive oil and cocoanut oil were chosen as solvents because they among the common oils represented as wide a variation in composition as could be obtained. Olive oil consists almost entirely of triolein, while cocoanut oil contains little olein, but large amounts of the saturated fatty acids (mainly lauric and myristic). Any difference in absorption due to difference in composition of the solvent oil would thus be noticed. The olive and cocoanut oils contained, as a rule, enough free fatty acid so that the solution emulsified well with dilute sodium carbonate. In those cases where it did not, enough oleic acid was added to produce the desired effect. The hydrocarbon oil solution was

<sup>9</sup> Bradley: *Proc. of the Amer. Soc. of Biol. Chem.*, Baltimore, 1911.

liquid at room temperature, the vaseline solution melted at about 30°C.

The experiments lasted six days each. The seventh day's feeding was the ordinary meat feeding together with bone ash, which served to separate the periods. Feces were collected daily. After the appearance of the bone-ash feces, the cages were scraped out and then rinsed with ether which was added to the extraction ether. The feces were mixed with acid alcohol and dried on a water bath. When dry they were powdered and extracted with ether in a Soxhlet extractor for twelve to sixteen hours.

The extracted fatty material was saponified with alcoholic alkali and the petroleum products separated with the unsaponifiable matter according to the method of Kumagawa-Suto. Mixtures of hydrocarbon oil with olive and cocoanut oils were carried through the processes of extraction and separation in order to determine the accuracy of the processes for these mixtures. Both the extraction and separation were found to be accurate.

Details and results of the individual experiments are given in the table below:

TABLE I.

EXPERIMENT NO.	MATERIAL FED	AMOUNT OF HYDROCARBON OIL FED	HYDROCARBON OIL RECOVERED FROM FECES	
			Grams	Per cent
		<i>grams</i>		
I	Hydrocarbon oil alone.....	17	15.9	93
II	Hydrocarbon oil alone.....	18	18.2	100
III	Hydrocarbon oil and olive oil	14.2	13.2	93
IV	Hydrocarbon oil and olive oil	22	20.2	92
V	Hydrocarbon oil and olive oil	22	20.0	91
VI	Hydrocarbon oil and cocoa-nut oil.....	16.5	14.5	88
VII	Hydrocarbon oil and cocoa-nut oil.....	18.6	16.9	85.5
VIII	Hydrocarbon jelly (vaseline) and olive oil.....	16.0	16.4	100
IX	Hydrocarbon jelly (vaseline) and olive oil.....	17.5	17.3	98.8

As may be seen from the table, the recovery of the hydrocarbon jelly (vaseline) was practically complete as was also that of the hydrocarbon oil when fed alone, while there was a slight discrepancy in the recovery of the hydrocarbon oil when fed in solu-

tion in olive and cocoanut oils which may have been due to absorption. Taking into account, however, the error of the experiment, the amount absorbed was probably negligible. The results with the hydrocarbon jelly bear out the findings of Henriques and Hansen<sup>10</sup> who after feeding solutions of vaseline to rats, found all (95 per cent) of the vaseline in the feces.

Since special precautions were taken not only to have the hydrocarbon products in solution in a readily absorbable oil, but also to have the mixture of such a composition that it would emulsify readily with dilute sodium carbonate solutions, the possibility of absorption of these substances either in solution in fats or fatty acids or in the form of emulsions is shown to be extremely unlikely.

*B. Chyle experiments with unemulsified (but emulsifiable)  
mixtures of the hydrocarbons.*

In continuation and extension of the feeding experiments a further series of experiments was conducted in which after feeding the same oil mixtures as before, the absorbed fat was collected by cannula from the thoracic duct and examined for absorbed hydrocarbons. The operations for the insertion of the cannula into the thoracic duct were done with aseptic precautions,<sup>11</sup> making

<sup>10</sup> Henriques and Hansen: *loc. cit.*

<sup>11</sup> It was believed that the ordinary method for collecting the chyle with the animal under the influence of an anaesthetic does not give the best results. The fat content of the chyle diminishes continuously from the beginning of collection, and if the stomachs of the animals are examined after death they are found to be distended with gas and to contain much of the material fed, while the intestines are empty. Digestion and absorption apparently cease after the animal has been under the anaesthetic for a time. It was thought therefore that a better digestion and a consequent greater richness of the chyle would result if the chyle could be collected after allowing the animal to recover from the anaesthetic. Attempts were therefore made to devise some means whereby they could be confined for this purpose. The attempts were mainly unsuccessful and the animals had to be reanaesthetized. Occasionally in the struggling the cannula would be pulled out and if it could not be inserted again the chyle was collected by pipette as it filled the wound cavity. In Experiment III the animal was successfully confined by the use of slings and, as may be seen from the protocol of the experiment, there was an improvement in the fat content of the chyle (as judged by its color) beginning about an hour after recovery from the anaesthetic.

a small opening and avoiding unnecessary injury to the tissues. By the use of a well paraffined cannula of narrow lumen (2 mm. or less) clotting of the chyle in the tube was entirely prevented. After the operation the wound was closed by suture. In the experiments below (with the exception of Experiment III), the chyle was collected in the ordinary way with the animal in ether anaesthesia. After collection and measurement, the chyle was transferred to a separatory funnel, saturated with potassium sulphate and extracted with ether over night. The ether solution was separated and the clear lymph evaporated to dryness, powdered and extracted with warm ether. The ether extract from the dried chyle was added to the first, the combined extract washed with water and then evaporated to dryness. The chyle fat was saponified with alcoholic potash and the unsaponifiable matter (which would contain the hydrocarbon oils) separated according to the method of Kumagawa-Suto.

**EXPERIMENT I.** *Hydrocarbon oil and olive oil, equal parts by weight.* A dog, weight 13 kgms., in good condition, having fasted for 48 hours, was fed 25 grams of the mixture at 8.45 a.m. The operation was begun at 11.15. Chyle was collected from 12.10 to 6 p.m. 12.10-1.30, 55 cc., milk-white; 1.30-2.45, 40 cc., becoming thinner; 2.45-4.00, 20 cc., as above; 4.00-4.45, 20 cc., thin; 4.45-6.00, 30 cc., almost transparent. Total, 165 cc. The chyle was extracted in three parts: (a) the first 55 cc., (b) the 110 cc. following, and (c) the dried chyle from (a) and (b). Total fat, 1.2 grams or 0.73 per cent of the amount of chyle. The fatty acids from this chyle fat melted at 30-32°C.

The analytical data of this and the succeeding experiments are given in Table II below.

**EXPERIMENT II.** *Hydrocarbon oil and olive oil, equal parts by weight.* A dog, weight 9 kgms., lean and active, after fasting for 48 hours, was fed 25 grams of the oil mixture at 8.45 a.m. The operation was begun at 11.15 and the cannula was in the duct and collection begun at 12.15. The duct ruptured at 12.30 and from then until 5 p.m. the chyle was collected by pipette as it flowed into the wound cavity. The chyle fat was extracted and the unsaponifiable matter separated in the regular way.

**EXPERIMENT III.** *Vaseline and olive oil, equal parts by weight.* A dog, weight 9 kgms., plump and in good condition, was fasted for 48 hours and fed 16 grams of the mixture at 9 a.m. Collection of the chyle began at 12.30. At 1.40 the animal was suspended in slings and allowed to recover from the anaesthetic. By 2.40 the effects of the ether had passed off and there was an immediate improvement in the fat content of the chyle, which lasted for about two hours. Chyle was collected for five hours. 12.30-1.40, 25 cc., white and milky; 1.40-2.40, 13 cc., becoming clear; 2.40-3.40, again milky; 3.40-4.40, 15 cc., milky; 4.40-5.40, 12 cc., again becoming

clear. The animal was becoming very restless and the experiment was concluded. Total chyle, 80 cc. The fatty acids from this chyle fat were solid at room temperature.

The analytical data are given in Table II below.

EXPERIMENT IV. *Vaseline and olive oil, equal parts by weight.* A dog, weight 11 kgms., after the usual fasting period, was fed 25 grams of the mixture at 8.45 a.m. Chyle was collected from 1 p.m. till 5.45 p.m., then extracted as before. The fatty acids from the chyle were liquid at room temperature.

EXPERIMENT V. *Hydrocarbon oil and coconut oil, equal parts by weight.* A dog, weight 9.5 kgms., thin, after a 48 hours' fast, was fed 34 grams of the mixture at 8.45 a.m. Collection was begun at 11.45 a.m. and continued until 5.45 p.m. The chyle, which at first was very rich in fat, gradually became less rich until at the end of the experiment it was almost transparent. (This diminution in the fat content of the chyle has been found to be characteristic of animals kept under ether anaesthesia for several hours and indicates a cessation of digestion, which is confirmed by post-mortem examination. The stomach is found to be distended with gas and to contain undigested material while the intestines are empty.) 11.45-1.25, 30 cc., rich and creamy; 1.25-2.25, 18 cc., milky; 2.25-3.25, 15 cc.; 3.25-4.40, 16 cc.; 4.40-5.50, 10 cc. Total chyle, 90 cc.

The analytical data are given in Table II.

TABLE II.

*Chyle experiments with mixtures of petroleum hydrocarbons and oils.*

CHYLE		CHYLE FAT		UNSAPONIFIABLE MATTER*		
Time	Volume cc.	Amounts grams	Per cent of chyle	Amounts gram	Per cent of chyle fat	M. P. °C.
EXPERIMENT I. <i>Hydrocarbon oil and olive oil fed.</i>						
12.30-1.30	55	0.5	1.1	0.032	6.4	112
1.30-6.00	110	0.4	0.55	0.03	7.5	
	Dried	0.3		0.03	10.0	
EXPERIMENT II. <i>Hydrocarbon oil and olive oil fed.</i>						
12.15-5.00	65	0.8	1.2	0.048	6.0	132
EXPERIMENT III. <i>Vaseline and olive oil fed.</i>						
12.30-5.40	80	1.6	2.0	0.05	3.2	110
EXPERIMENT IV. <i>Vaseline and olive oil fed.</i>						
1.00-5.45	75	0.5	0.7	0.025	5.0	112
EXPERIMENT V. <i>Hydrocarbon oil and cocoanut oil fed.</i>						
1.45-5.50	90	1.2	1.3	0.063	5.3	110

\* The unsaponifiable matter was crystalline in all cases.

From the data given above it may be seen that the unsaponifiable fraction (which would have contained the hydrocarbon products if present) is small in amount—mostly less than 7 per cent—and hence falls within the limits for unsaponifiable matter in the fat of normal dog chyle.<sup>12</sup> It is crystalline, its melting point is high (110°C. or over) and it gives the tests for cholesterin, from which facts we may conclude that the unsaponifiable matter consists, as normally, mainly of cholesterin. There is therefore no appreciable absorption of the hydrocarbon oils into the chyle. The results are thus in agreement with the results of the feeding experiments already reported, but are contrary to those of Bradley<sup>13</sup> who found after feeding an emulsified mixture of equal parts of hydrocarbon oil and the olive oil that the chyle fat contained both substances in about the same proportions as fed.

*C. Feeding of emulsions of the petroleum oils and examination of the chyle fat.*

In Bradley's experiments the petroleum oil was fed as an actual emulsion and although there is little reason to believe that such an emulsion would behave differently from the emulsion formed normally in the intestine, a third series of experiments was conducted in which thoroughly emulsified mixtures of hydrocarbon oil and olive oil were fed and the chyle fat examined for hydrocarbon oils as before. Lecithin and gum acacia were used as emulsifying agents because of the resistance of emulsions formed with them to the action of the gastric juice.

The lecithin emulsion was made by dissolving 5 grams of lecithin (Merck) in 50 cc. of water and adding 45 grams of a mixture of equal parts of hydrocarbon oil and olive oil in small portions with continuous shaking, the whole operation taking about one hour. The emulsion so prepared showed no signs of separation of the oil in three days.

The acacia emulsion was prepared according to directions in the *National Formulary* with the same oil mixture as above, and then diluted with water until it would readily pass through a stomach

<sup>12</sup> Munk: *Virchow's Archiv*, cxiii, p. 230.

<sup>13</sup> Bradley: *loc. cit.*



tube. The emulsion showed no signs of disintegration in the course of a week.

After a preliminary fasting period the dogs were fed with the emulsions and about two hours later the operation for insertion of the cannula into the thoracic duct was performed. A new procedure was adopted with the intention of securing a better and more natural flow of chyle. As soon as the operation was completed the anaesthetic was stopped, the wound closed by sutures, the animal removed to a well-padded table and secured on his side so that the chyle from the cannula would drop into a beaker. As soon as the effects of the anaesthetic had passed off the animals generally ceased struggling and frequently went to sleep. Water was given as soon as and as often as they would take it. An improvement in the fat content of the chyle was noted generally within two hours after placing on the table. Chyle was collected as long as convenient or until the dog became restless. Before removal to the cage a broad metal collar with a slot for the cannula was fitted on. The lymph was in most cases still flowing on the second day and occasionally a second experiment could then be done with the animal. The cannula generally came out on the third day, after which the wound healed and the dog in the course of a short time was none the worse for the experiment.

**EXPERIMENT I.** *Lecithin emulsion of equal parts of hydrocarbon oil and olive oil.* A dog, young and active, weight 7.5 kgms., after a preliminary fasting for 48 hours was fed 50 grams of the lecithin emulsion (containing 22.5 grams of the oil mixture) at 10 a.m. and operated on at 12.00 m. The cannula was in the duct and collection begun at 1 p.m. As soon as the cannula was secured in the duct the wound was closed by sutures and the animal removed to a padded table, covered warmly and allowed to recover from the anaesthetic. Water was given as desired. The chyle was collected in a beaker as it dropped from the cannula. At first it was almost transparent, but in the course of two or three hours, as the animal recovered from the anaesthetic, the fat content improved and continued good to the end of the period of collection. Chyle was collected for eight hours, during which time the animal rested quietly—much of the time asleep. 1–2 p.m., 40 cc., poor in fat; 2–3 p.m., 15 cc., poor in fat; 3–4 p.m., 10 cc., milky; 4–5 p.m., 14 cc., milk-white; 5–6 p.m., 30 cc., milk-white; 6–7 p.m., 22 cc., cream-like; 7–8 p.m., 22 cc., as before; 8–9 p.m., 26 cc., as before. Total chyle, 179 cc. Next morning the chyle was still flowing and was almost water-clear so another feeding of emulsion was given, but before

the time for collection the flow ceased. The chyle was extracted in two portions: (1) the first 65 cc., (2) the remainder (114 cc.), after which the two portions were united, evaporated to dryness and the dry material extracted. The extracted fat was saponified and the unsaponifiable matter separated as before. The fatty acids<sup>14</sup> from this fat melted at 29.5°.

The analytical details of the experiment are given in the table (III) below.

**EXPERIMENT II.** *Acacia emulsion of hydrocarbon oil and olive oil.* A dog, old, but in good condition, was fasted for 48 hours, then at 8.45 a.m., fed an acacia emulsion of 35 grams of the above oil mixture. The operation for insertion of the cannula was performed as usual and the collection of the chyle begun at 12.15. The animal was made comfortable on the padded table and as soon as he had recovered from the anaesthetic was given water freely. This dog struggled considerably and the flow of chyle lasted only six hours. 12.15–1 p.m., 23 cc., rich in fat; 1–2, 15 cc., rich in fat; 2–3, 22 cc., milk-white; 3–4, 18 cc., fat content decreasing; 4–5, 14 cc., thin; 5–6.30, 8 cc., almost transparent. Total chyle, 100 cc. The fatty acids melted at 30°C.

The analytical details of the experiment are given in the table below.

TABLE III.

*Chyle experiments with emulsions of the hydrocarbon oil.*

CHYLE		CHYLE FAT		UNSAPONIFIABLE MATTER		
Time p.m.	Volume cc.	Amounts grams	Per cent of chyle	Amounts gram	Per cent of chyle fat	M. P. °C.
EXPERIMENT I. <i>Hydrocarbon oil and olive oil, lecithin emulsion.</i>						
1.00-4.00	65	0.3	0.47	3.3	0.095	1.7
4.00-9.00	114	4.6	5.9			
	Dried	1.0	4.0			
EXPERIMENT II. <i>Acacia Emulsion.</i>						
12.15-6.30	100	4.1	4.1	0.086	2.1	120

The fat absorption in these experiments was very good—52 per cent in 8 hours and 24 per cent in 6 hours<sup>15</sup> respectively—indicating that the processes of saponification and absorption of the fats were proceeding in a nearly if not quite normal manner.

In these experiments, as in the preceding, the amount and nature of the unsaponifiable matter precludes the possibility of the ab-

<sup>14</sup> A more detailed examination of this material is being made and will be reported in a later publication.

<sup>15</sup> Actually, in four hours, since there was practically no fat in the chyle of the last hour and a half.

sorption of any appreciable amount of the hydrocarbon oil. The results, while contrary to those of Bradley, are in agreement with all other reported results with the petroleum hydrocarbons and give no grounds for the belief that these substances are absorbed from the intestine.

## II. THE ABSORPTION OF WOOL-FAT.

The hydrocarbon oils are substances which are never normally present in the tissues of animals and probably cannot be utilized by them. Their failure of absorption may have been due to a protective mechanism acting to prevent the absorption of fat-like substances which cannot be utilized. Cholesterin and its fatty acid esters on the other hand are constant constituents of the blood and other tissues and are therefore presumably useful substances. If it be found, as the experiments of Connstein indicate, that these substances are not absorbed, their rejection would be on a different basis from that of the hydrocarbon oils. The cholesterin esters of wool-fat, while they form excellent and finely divided emulsions with water and dissolve in fats and fatty acids, are not saponified by the lipases of the intestinal tract. Consequently if absorption depended on water solubility with hydrolysis as a necessary preliminary, these substances would be rejected, while if there were any absorption as emulsions or in solution in fats, they would be absorbed.

In the following experiments wool-fat (Merck's Lanolin) was fed to cats together with an adequate diet of boiled lean meat, free from fat. In order to make sure that the melting point of the wool-fat as fed was well below body temperature, as well as to get the benefit of any solvent effect of ordinary fats or fatty acids during absorption, the wool-fat was fed in solution in an equal weight of olive oil.

The experiments lasted six days each with an extra day in which bone ash was fed with the meat instead of the wool-fat. At the end of each period the feces were dried, ground and extracted with ether in a Soxhlet extractor for sixteen hours. Since it was not possible to separate the wool-fat from the ordinary feces fat, a three weeks' fore period was conducted on the diet without the wool-fat, and the average fat excretion for one week for each

animal determined. This was taken as the basal fat excretion and the difference between the total fat excretion on the wool-fat diet and this basal fat excretion was considered as unabsorbed wool-fat. The experiments resulted as follows:

EXPERIMENT I. *Fed 16.8 grams of wool-fat.*

Total ether extract of feces.....	17.1 grams.
Basal fat excretion.....	1.3 grams.
<hr/>	
Wool-fat recovered.....	15.8 grams, 94 per cent.

EXPERIMENT II. *Fed 17.8 grams of wool-fat.*

Total ether extract of feces.....	20.4 grams.
Basal fat excretion.....	2.1 grams.
<hr/>	
Wool-fat recovered.....	18.3 grams, 100 per cent.

The results thus bear out those of Connstein and show that wool-fat is not absorbed from the intestine. Since these esters differ from ordinary fats and other absorbable esters chiefly in that they cannot be saponified by the intestinal lipases, their rejection is in all probability due to that fact.

SUMMARY AND CONCLUSIONS.

In the foregoing experiments the absorption of two classes of fat-like substances, petroleum hydrocarbons and unsaponifiable esters (wool-fat), has been investigated. None of them were found to be absorbed.

The substances as fed were similar to ordinary fats in most of their properties. They emulsified well with dilute alkalies, were soluble in fats and fat solvents, and melted below body temperature. They differed from the fats mainly in that they could not be reduced to water-soluble form in the intestine.

The slow passage of the fats from the stomach, the abundant provision for hydrolysis and for the absorption of the products of hydrolysis in the intestine and the failure of absorption of fat-like substances which cannot be changed to a water-soluble form, make it extremely probable that fats can be absorbed only in water-soluble form and that saponification is a necessary preliminary to absorption.

The significance of this mechanism is little understood but in the light of the above results, one of its uses would appear to be to exclude undesirable fat-like substances which would otherwise be carried in with the fats.

The operations for insertion of the cannula into the thoracic duct were performed by Dr. W. McK. Marriott of this laboratory, to whom I take this opportunity of expressing my indebtedness.



## RESEARCHES ON PURINES. XI.<sup>1</sup>

### ON 2,8-DIOXY-6-METHYL-9-ETHYLPURINE.

By CARL O. JOHNS AND EMIL J. BAUMANN.

(From the Sheffield Laboratory of Yale University.)

(Received for publication, May 19, 1913.)

Although a large number of purines containing methyl groups are known, but very few purines containing ethyl groups have been described. Sembritzki,<sup>2</sup> working according to the method of Fischer and Ach,<sup>3</sup> prepared 1,3-diethylpseudouric acid from which he obtained 1,3-diethyluric acid. By means of the same method, Armstrong<sup>4</sup> synthesized 9-ethylpseudouric acid (VIII) and from this compound he prepared 9-ethyluric acid (IX). He also found that the latter compound could be alkylated further by means of ethyl iodide and in this manner he obtained a diethyluric acid of unknown constitution.

The method of preparing the 9-ethylpurine derivative, which we describe in this communication, is similar to that already used in this laboratory for the preparation of 9-methylpurines,<sup>5</sup> namely, to first obtain an orthodiaminopyrimidine in which a hydrogen atom of the amino group in position 6 (or 4) has been replaced by an ethyl group (V).

The reactions employed in the synthesis of 2,8-dioxy-6-methyl-9-ethylpurine (IV) are as follows: 2-Ethylmercapto-4-methyl-6-chlorpyrimidine (I)<sup>6</sup> was heated with aqueous ethylamine and gave an excellent yield of 2-ethylmercapto-4-methyl-6-ethylaminopyrimidine (II). The latter compound was then boiled with hydrochloric acid and ethylmercaptan was evolved freely. The resulting

<sup>1</sup> Johns and Baumann: this *Journal*, xiv, p. 381, 1913.

<sup>2</sup> Kurt Sembritzki: *Ber. d. deutsch. chem. Gesellsch.*, xxx, p. 1814, 1897.

<sup>3</sup> Fischer and Ach: *ibid.*, xxviii, p. 2473, 1895.

<sup>4</sup> E. Frankland Armstrong: *ibid.*, xxxiii, p. 2308, 1900.

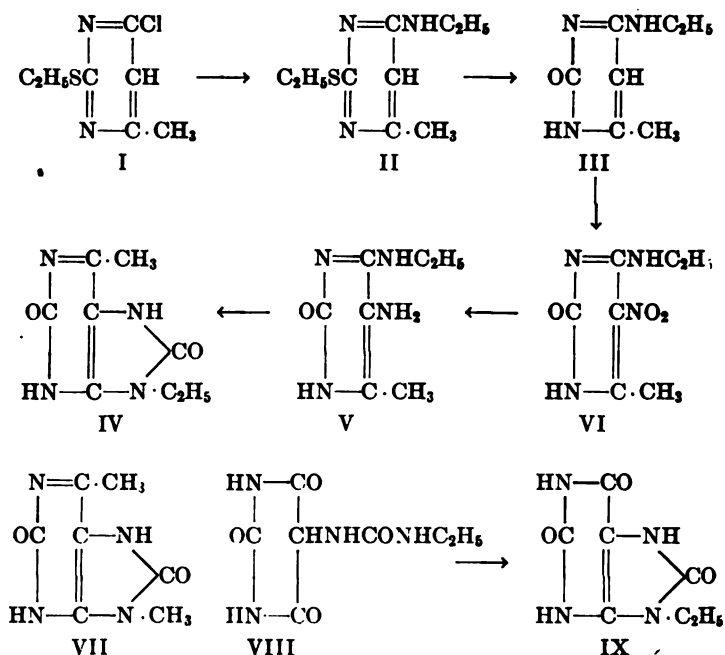
<sup>5</sup> Johns: this *Journal*, ix, p. 161, 1911.

<sup>6</sup> Johns: *Amer. Chem. Journ.*, xl, p. 351, 1908.

2-oxy-4-methyl-6-ethylaminopyrimidine (III) gave a nitro compound (VI) which could readily be reduced to 2-oxy-4-methyl-5-amino-6-ethylaminopyrimidine (V) by using freshly precipitated ferrous hydroxide as the reducing agent. A very good yield of 2,8-dioxy-6-methyl-9-ethylpurine (IV) was obtained by heating the 2-oxy-4-methyl-5-amino-6-ethylaminopyrimidine with urea.

Since 2,8-dioxy-6-methyl-9-ethylpurine (IV) is a homologue of 2,8-dioxy-6,9-dimethylpurine (VII),<sup>7</sup> we would expect the properties of these purines to be closely related, and we find this to be the case.

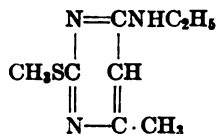
These researches will be continued.



<sup>7</sup>Johns: this *Journal*, xi, p. 397, 1912.

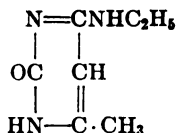


## EXPERIMENTAL PART.

*2-Ethylmercapto-4-methyl-6-ethylaminopyrimidine.*

Twenty grams of 2-ethylmercapto-4-methyl-6-chloropyrimidine\* were mixed with 28.8 grams of a 33 per cent aqueous solution of ethylamine and the mixture heated in a sealed tube at 90°–100°C. for three hours. The reaction product was obtained in the form of a heavy yellow oil. This was drawn off in a separatory funnel and washed with water. When the oil was scratched with a glass rod in a beaker it solidified to a crystalline mass. The yield was 20 grams or 95 per cent of the calculated amount, after being dried at room temperature. The substance was very soluble in alcohol, ether or benzene. It was also soluble in cold dilute acids but almost insoluble in dilute alkalies. It was slightly soluble in hot water, and on cooling the solution, it was precipitated as an oil. It was very soluble in boiling ligroin, and crystallized well in the form of colorless pointed prisms on cooling the solution slowly. The crystals thus obtained melted at 70°C.

	Calculated for $\text{C}_9\text{H}_{11}\text{N}_3\text{S}$	Found:
N .....	21.32	21.36

*2-Oxy-4-methyl-6-ethylaminopyrimidine.*

Twenty grams of 2-ethylmercapto-4-methyl-6-ethylaminopyrimidine were dissolved in 100 cc. of concentrated hydrochloric acid, the solution was boiled gently under a reflux condenser for 1 hour. Ethylmercaptan was evolved rapidly. The solution was evaporated to dryness on a steam bath. This treatment left

\* *Amer. Chem. Journ.*, xl, p. 351, 1908.

a hydrochloride of 2-oxy-4-methyl-6-ethylaminopyrimidine. This salt was dissolved in water and the solution was made slightly alkaline with ammonium hydroxide. On concentrating on the steam bath, clusters of crystals appeared on the surface of the liquid. The evaporation was then discontinued and the solution was cooled slowly. A bulky mass of crystals was obtained. The crystals were filtered off by suction and washed with a little cold water to remove ammonium chloride. The yield was 11.5 grams or 75 per cent of the calculated. A considerable amount remained in the mother liquor. The compound was very soluble in hot and moderately soluble in cold alcohol. It was insoluble in ether and slightly soluble in benzene. It was easily soluble in dilute acids and alkalies. It was very soluble in hot water and moderately soluble in cold water and from this solvent it crystallized in acicular prisms. These melted partially and decomposed at 245°–250°C.

	Calculated for $C_7H_{11}ON_3$ :	Found:
N.....	27.45	27.25

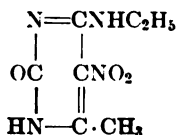
*The hydrochloride of 2-oxy-4-methyl-6-ethylaminopyrimidine.*



This compound was prepared by dissolving 1 gram of the base in 5 cc. of 20 per cent hydrochloric acid and concentrating until crystals could be obtained on cooling. When such a solution was cooled slowly, colorless diamond-shaped plates were obtained. These were filtered off and washed with concentrated hydrochloric acid and dried at about 80°C. They were very soluble in water. They melted to a clear oil at 214°–215°C. The yield was only about 0.2 gram from 1 gram of the base as the hydrochloride was soluble in dilute hydrochloric acid.

	Calculated for $C_7H_{11}ON_3 \cdot HCl$ :	Found:
Cl.....	18.73	18.86

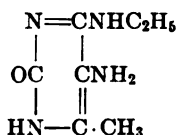
*2-Oxy-4-methyl-5-nitro-6-ethylaminopyrimidine.*



Five grams of 2-oxy-4-methyl-6-ethylaminopyrimidine were dissolved in 10 cc. of cold concentrated sulphuric acid. Heat was evolved as solution took place but the temperature was kept down to 60°–70°C. To the still warm solution were then added gradually 5 cc. of nitric acid, density 1.5. Heat was generated. When the addition of nitric acid was complete, the resulting solution was kept at 60°–70° for five minutes, and then poured into cold water. This solution was cooled and cautiously neutralized with concentrated ammonium hydroxide. As soon as an excess of ammonia was present, a yellow color appeared. This was removed by acidifying slightly with acetic acid. The white precipitate which formed, was filtered off by suction and washed with cold water and dried at 90°–100°C. The yield was 5.9 grams, which corresponds to 91 per cent of the calculated weight. The nitro compound dissolves in hot ammonium hydroxide and a yellow ammonium salt crystallizes out on cooling. It also dissolves in dilute alkalies forming yellow solutions. It is slightly soluble in hot alcohol or benzene and insoluble in ether. It dissolves sparingly in hot water and from this solvent it crystallizes in needles. It dissolves in dilute hydrochloric acid and in glacial acetic acid at room temperature. It begins to decompose slowly at about 238°C. and chars at 260°–265°C.

	Calculated for $C_7H_{10}O_2N_4$	Found:
N.....	28.28	28.14

*2-Oxy-4-methyl-5-amino-6-ethylaminopyrimidine.*



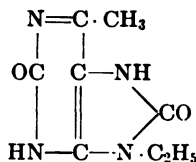
Five grams of 2-oxy-4-methyl-5-nitro-6-ethylaminopyrimidine were suspended in 100 cc. of water on a steam bath and 75 cc. of concentrated ammonium hydroxide were added. The nitro-pyrimidine dissolves on warming gently. A hot concentrated solution of 50 grams of crystallized ferrous sulphate was added. Reduction proceeded rapidly and was accompanied by the liberation of heat. The sulphate was precipitated by the addition of a concentrated solution of 60 grams of barium hydroxide and after thorough

shaking the excess of baryta was precipitated by the addition of ammonium carbonate. The resulting mixture was kept warm for about one-half hour and then filtered. The precipitate was thoroughly washed with hot water. The filtrate and washings were then concentrated to a small volume, clarified once with blood coal and then concentrated to about 10 cc. About 3 grams of crystals were obtained on cooling the solution. More of the diamino-pyrimidine was obtained from the mother liquor, the total yield being 90–95 per cent of the calculated amount. The crystals were not soluble in ether and but slightly soluble in benzene. They were very soluble in hot alcohol or hot water and moderately soluble in cold water. They dissolved readily in cold alkalis or acids. An aqueous solution did not give a precipitate on adding barium chloride and did not form a difficultly soluble picrate. Mercuric chloride produced a white precipitate which became dark when the solution was boiled. An ammoniacal silver solution was reduced in the cold with the formation of a silver mirror. The substance crystallized from water in clusters of needles that contained one molecule of water of crystallization after drying over sulphuric acid. On drying for two hours at 130°C. the crystals became anhydrous.

1.8828 grams of substance lost 0.1803 gram of  $H_2O$ .

	Calculated for $C_7H_{12}ON_4 \cdot H_2O$ :		Found:
$H_2O$ .....	9.67		9.57
	Calculated for $C_7H_{12}ON_4$ :		Found:
N .....	33.33	I 33.36	II 33.41

*2,8-Dioxy-6-methyl-9-ethylpurine.*



One gram of 2-oxy-4-methyl-5-amino-6-ethylaminopyrimidine and 1 gram of urea were pulverized together in a mortar, and the mixture was heated in an oil bath at 170°–180°C. for an hour. The

urea melted, leaving the pyrimidine partly suspended in the liquid, but on heating for about a half hour, the whole mass began to solidify. Very little charring occurred. The reaction product was dissolved in hot dilute ammonium hydroxide, producing a red solution. This was clarified with blood coal. The resulting solution was acidified with acetic acid whereupon the purine crystallized out. On cooling, filtering and washing out the salts with water, 0.9 gram of pure purine was obtained. This is 78 per cent of the calculated amount. The purine dissolved in about 35 parts of boiling water and in about 500 parts of water at room temperature. It was not soluble in ether and but slightly soluble in boiling benzene or alcohol. It dissolved readily in dilute alkalies and was moderately soluble in cold dilute acids. It crystallized from water in needles that formed sheaves like tyrosine. It did not form a difficultly soluble picrate and did not give a precipitate with mercuric chloride or barium chloride. When added to an ammoniacal silver solution it formed a jelly which did not seem to be changed by heating. When heated with concentrated nitric acid on the steam bath it oxidized and left a yellow film, but if sufficient purine was present, a blood-red residue was obtained. The yellow film turned red when it was moistened with ammonia and dried on the steam bath. The crystallized purine possessed a pearly lustre and was found to be anhydrous. It did not melt at 310°C.

0.2073 gram of substance gave 0.3790 gram of  $\text{CO}_2$  and 0.0942 gram of  $\text{H}_2\text{O}$ .

	Calculated for $\text{C}_8\text{H}_{10}\text{O}_2\text{N}_4$	Found:	
C.....	49.48	49.86	
H.....	5.04	5.08	
N.....	28.87	<sup>I</sup> 29.14	<sup>II</sup> 29.08



# THE INTERCONVERSION OF $\alpha$ -AMINO-ACIDS, $\alpha$ -HYDROXY-ACIDS AND $\alpha$ -KETONIC ALDEHYDES.

## PART II.

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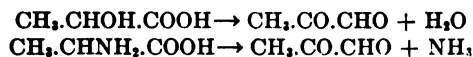
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### 1. Introduction.

The object of the following paper is to present the detailed experiments upon which we have based a hypothesis concerning the intermediary metabolism of amino- and hydroxy-acids, and in particular the mechanism concerning the mutual interconversion of alanine, lactic acid and glucose.<sup>1</sup> For the sake of clearness, we may reproduce the essential features of the types of reactions which we believe to be operative in the changes concerned.

By making use of a substance capable of forming extremely insoluble derivatives with  $\alpha$ -ketonic aldehydes, namely, para-nitrophenylhydrazine, we have been able to show, by experiments *in vitro*, that amino-acids and hydroxy-acids, such as alanine and lactic acid, readily undergo decomposition in faintly acid solution in conformity with the following equations:

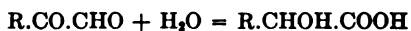


<sup>1</sup> This *Journal*, xiv, p. 555, 1913.

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Amino-acids have been commonly regarded as extremely stable substances, at least *in vitro*, but our observations tend to show that under suitable conditions, in aqueous solution, when due provision is made for the prompt removal of the products of their decomposition, both the  $\alpha$ -amino-acids and  $\alpha$ -hydroxy-acids are in a state of unstable equilibrium.<sup>2</sup> Furthermore, we have been able to show that the decomposition of amino-acids with formation of ketonic aldehydes is not due to a complicated reaction dependent upon the presence of the nitrophenylhydrazine, for it has been possible to demonstrate ammonia formation from amino-acids under similar conditions, but in the absence of the hydrazine.

The production of  $\alpha$ -ketonic aldehydes from  $\alpha$ -amino- and  $\alpha$ -hydroxy-acids is of biochemical significance, partly on account of the existence of enzymes which we have named "glyoxalases" capable of converting the former substances into hydroxy-acids.<sup>3</sup>



Moreover, we have been able to gather a considerable amount of indirect evidence indicating that  $\alpha$ -ketonic aldehydes may play a part in intermediary metabolism. Thus we find that methyl glyoxal yields glucose in the glycosuric organism just as do alanine and lactic acid, from which methyl glyoxal may be derived *in vitro* (section 7).

In addition, it is possible to demonstrate, *in vitro*, the reverse change, namely, the conversion of glucose into methyl glyoxal under conditions which, apart from temperature, are comparable with those existing in the animal body (section 4).

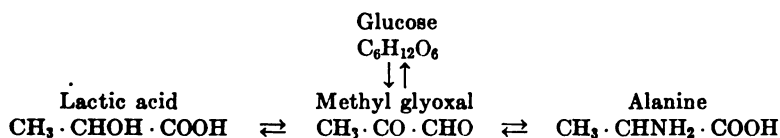
From the foregoing evidence and other that has been referred to in our previous paper, it appeared justifiable to construct a scheme which may crudely represent the interconversion of alanine, lactic acid, methyl glyoxal and glucose by a series of reversible reactions involving the addition or subtraction of water or ammonia. Of the various reactions indicated, the direct formation of alanine from methyl glyoxal is the only one that thus far has

<sup>2</sup> It is of interest to note that the  $\beta$ -amino-acids, such as  $\beta$ -alanine and  $\beta$ -phenylalanine, which do not occur in nature, do not yield ketonic aldehydes, at any rate under the above conditions.

<sup>3</sup> This *Journal*, xiv, p. 423, 1913.

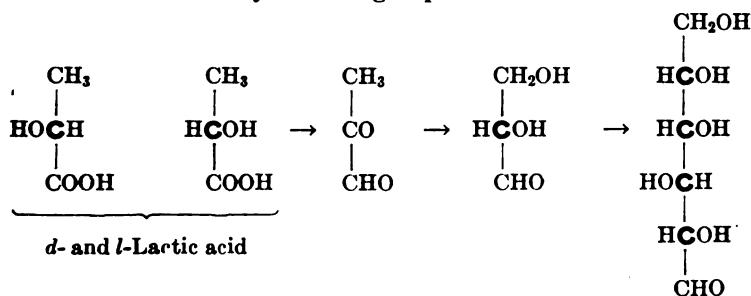


not been demonstrated although the analogous synthesis of glycine from glyoxal has been effected.



The lactic acid which we have obtained by the action of glyoxalase upon methyl glyoxal is a mixture of the two forms in which the laevo acid is in excess. The production of glucose from *d*-lactic acid and from the inactive acid is well established through Mandel and Lusk's experiments, but it appeared very desirable to determine whether the pure laevo acid might also yield glucose. Accordingly, we have prepared pure *l*-lactic acid from morphine *l*-lactate and find that it also yields glucose freely in the glycosuric animal.

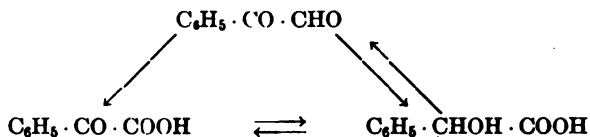
This result appears to us to be of considerable significance, for the almost quantitative conversion of both *d*- and *l*-lactic acids, substances possessing asymmetric carbon atoms enantiomorphously related, into the same *d*-glucose apparently necessitates a loss of asymmetry in the lactic acid molecule in the process of glucose synthesis. The intermediate formation of methylglyoxal, such as we have suggested, would furnish a satisfactory explanation of such a change, and in addition, the conversion of methyl glyoxal into glucose, possibly with intermediate formation of glyceric aldehyde, would give an opportunity for the introduction of new asymmetric groups.



Finally, reference may be made to the relation of the  $\alpha$ -ketonic acids to amino-acids and  $\alpha$ -ketonic aldehydes. Neubauer and

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Knoop have clearly demonstrated the interconversion of amino- and ketonic acids. A clue to the mechanism of this reaction may be furnished by our observations (section 6) on the formation of phenyl glyoxylic acid as well as *l*-mandelic acid on perfusing a liver with blood containing phenyl glyoxal.



It is possible that the phenyl glyoxylic acid originates as a secondary product of the oxidation of mandelic acid rather than by the direct oxidation of phenyl glyoxal.<sup>4</sup> But in any case, the result is of interest since it serves to bring the  $\alpha$ -ketonic aldehydes in close biochemical relation with the amino- as well as the hydroxy-acids.

In the experimental part of this paper it will be shown that the nitrophenylhydrazones of glyoxylic and pyruvic acids may be obtained by the direct action of nitrophenylhydrazine upon glycollic and lactic acids. Hydrazino-acids appear to be first formed and are readily oxidized to the ketonic acid derivatives in the presence of air. Although ordinary hydrazines are not known to occur in the animal body many substances such as arginine and creatine contain the  $-\text{NH} \cdot \text{NH}_2$  group and it is conceivable that substances of this type may be concerned in the biochemical oxidation of hydroxy to ketonic acids.

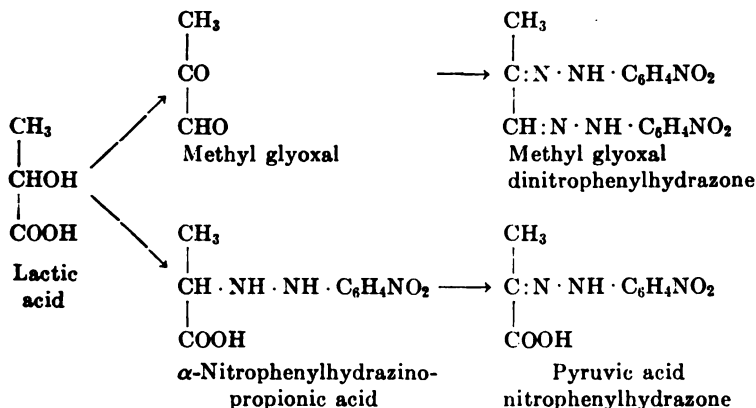
### 2. The formation of methyl glyoxal from lactic acid.

It is an extremely easy matter to demonstrate the formation of methyl glyoxal from lactic acid by simply allowing a filtered 5 or 10 per cent aqueous solution of lactic acid (500 cc.) containing a little nitrophenylhydrazine (1-2 grams) to stand at room temperature or in the incubator. After two or three hours, a flocculent red precipitate begins to appear, which in the earlier

<sup>4</sup> It is of interest to note that Evans (*Amer. Chem. Journ.*, xxxv, p. 128, 1906) oxidized phenyl glyoxal to phenyl glyoxylic acid by means of alkaline permanganate. In the absence of alkali benzoic acid is obtained.

stages of the reaction, is composed of almost pure methyl glyoxal dinitrophenylhydrazone.

The precipitate gradually increases in amount during the succeeding three or four days, when it will be found that all the nitrophenylhydrazine has disappeared, although the amount of precipitate does not account for nearly all of the base added.<sup>5</sup> When this stage has been reached, it is well to filter off the precipitate on a small funnel and to dissolve an additional quantity of nitrophenylhydrazine (1-2 grams) in the filtrate by warming and subsequently cooling and filtering from any trace of insoluble matter. The clear filtrate, on standing, soon begins to deposit more methyl glyoxal dinitrophenylhydrazone and the process may be repeated as often as desired. After a time it will be noticed, however, that the character of the precipitate begins to change and that a yellowish-brown crystalline substance begins to deposit in addition to the amorphous methyl glyoxal derivative. The crystalline deposit is a mixture of nitrophenylhydrazinopropionic acid and the nitrophenylhydrazone of pyruvic acid, the latter being formed from the former by oxidation. The changes may be represented as follows:



The separation of the constituents of the red precipitate may be conveniently carried out as follows:

The mixture is first of all washed with hot 10 per cent sodium

<sup>5</sup> Unchanged nitrophenylhydrazine is conveniently tested for by adding a drop of acetone or benzaldehyde to a few cubic centimeters of the solution.

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carbonate solution which dissolves the nitrophenylhydrazino-propionic acid and the pyruvic acid nitrophenylhydrazone. The residue is then washed with warm alcohol and finally dissolved in a small amount of boiling nitrobenzene, filtered hot and toluene added to the filtrate. The pure methyl glyoxal dinitrophenylhydrazone separates out almost immediately in the form of glistening crimson needles which are filtered off, washed with toluene and dried at 140°. The substance was identical in every way with the hydrazone prepared directly from methyl glyoxal and melted with decomposition at about 302°–304° after darkening slightly above 290°. On warming the merest trace of the substance with caustic soda solution, best with the addition of a few drops of alcohol, there develops a magnificent deep blue color slowly changing to purple, then violet and finally a dull brown-red. The reaction is extremely sensitive.

ANALYSIS: 0.1218 gram dried at 150° gave 0.0299 gram N = 24.6 per cent N.  
 $C_{15}H_{14}O_4N_6$  requires 24.6 per cent N.

For purposes of comparison, the nitrophenylhydrazone of methyl glyoxal was prepared directly from methyl glyoxal obtained by the hydrolysis of its acetal (Meisenheimer). A slight excess of the hydrazine (2.2 mols.) dissolved in 30 per cent acetic acid was added to methyl glyoxal (1 mol.). The hydrazone is at once precipitated in practically theoretical amount and may be washed with alcohol and then recrystallized as above from nitrobenzene and toluene. The substance is very sparingly soluble in almost all solvents with the exception of nitrobenzene and bases such as pyridine. It should be noted that nitrobenzene and especially pyridine, combine with the substance very tenaciously and are only given off *in vacuo* at 100° with extreme slowness. Heating at 140°–150° is much more efficient in driving off the solvents. Pyridine seems to be a somewhat more objectionable solvent than nitrobenzene on account of the ease with which it unites with the hydrazone forming dark red-brown solutions. The pure substance melts at 302°–304°, the exact temperature varying slightly with the speed of heating. On analysis the substance was found to contain 25.1 per cent nitrogen (theory = 24.6). The same substance has been described by Neuberg,<sup>6</sup> who obtained it from  $\alpha$ -aminopropionic

<sup>6</sup> *Ber. d. deutsch. chem. Gesellschaft.*, xli, p. 956, 1908.

aldehyde. The melting point is given as  $277^{\circ}$  with discoloration at  $255^{\circ}$ . We believe this melting point to be much too low. The reaction with caustic soda was not described.

The  $\alpha$ -nitrophenylhydrazinopropionic acid and pyruvic acid nitrophenylhydrazone contained in the sodium carbonate washings from the original precipitate were recovered on acidifying with acetic acid. On repeated recrystallization from boiling water, the first substance is slowly oxidized to the pyruvic acid derivative, so that probably the former was not obtained perfectly pure. It is fairly soluble in alcohol, melts above  $250^{\circ}$  and gives an intense red coloration with caustic soda.

ANALYSIS: 0.0919 gram substance gave 0.0169 gram N = 18.3 per cent N.  
 $C_8H_{11}O_4N_2$  requires 18.6 per cent N.

By repeated crystallization from water of the mixture of the hydrazino acid and the pyruvic acid nitrophenylhydrazone, several grams of the latter were readily obtained as a bright yellow crystalline substance melting at  $223^{\circ}$ – $225^{\circ}$ . It is also formed by oxidizing nitrophenylhydrazinopropionic acid with an ammoniacal solution of a cupric salt. It is moderately soluble in alcohol and gives a bright red color on addition of caustic soda.

ANALYSIS: 0.1225 gram gave 0.0228 gram N = 18.6 per cent N.  
 $C_8H_9O_4N_2$  requires 18.8 per cent N.

The substance obtained from lactic acid was identical with the product obtained from pyruvic acid as described by Hyde<sup>7</sup> and also prepared for comparison by us. The melting point given by Hyde is  $219^{\circ}$ – $220^{\circ}$ .

A number of experiments were made in which additions of other substances were made to the lactic acid mixture in the hope of accelerating its decomposition into methyl glyoxal and water. The following were tried: spongy platinum, aluminum oxide, chromium oxide, mercuric iodide, uranium acetate, glycine, sulphuric acid and calcium lactate. None of them proved effective.

It should be noted that while the yield of crystalline substances from the lactic acid is small, the greater part of the acid remains unchanged and may be treated over and over again with fresh nitrophenylhydrazine.

<sup>7</sup> *Berichte*, xxxii, p. 1815.

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### 3. *The formation of methyl glyoxal and ammonia from alanine.*

The formation of methyl glyoxal from alanine is readily demonstrated by allowing an aqueous solution of the amino-acid with a little nitrophenylhydrazine and a few drops of an acid to stand at room temperature, or better in the incubator at 39°. After a few hours, the separation of a red precipitate commences and its quantity gradually increases from day to day. From time to time it is advisable to filter off the precipitate and to add more nitrophenylhydrazine. If no acid be added to the mixture, a precipitate is still obtained but it contains little or none of the methyl glyoxal derivative. Comparative experiments in which equivalent amounts of sulphuric and acetic acids were used, failed to show any marked difference.

In one experiment, a filtered solution containing alanine (25 grams), nitrophenylhydrazine (1.5 grams) and acetic acid (5 cc.) and water (500 cc.) was digested at 39°. After three hours, the separation of a precipitate was noticeable and the amount gradually increased during the following four days, when almost all the nitrophenylhydrazine had disappeared. The precipitate was filtered off and additional nitrophenylhydrazine (1 gram) dissolved in the filtrate. A second precipitation soon commenced and the whole process was eventually repeated four times.

The combined precipitates which weighed rather less than a gram, were purified by washing successively with hot 10 per cent sodium carbonate solution, water and alcohol. The residue was then crystallized from a mixture of nitrobenzene and toluene and was obtained in the form of red needles melting at 302° identical with methyl glyoxal dinitrophenylhydrazone prepared from other sources. It gave the color reaction with caustic soda in typical fashion. On mixing the substance from alanine with a preparation from methyl glyoxal, the melting point of the mixture was unchanged.

ANALYSIS: 0.1367 gram gave 0.0333 gram N = 24.4 per cent N.  
 $C_{11}H_{14}O_4N_6$  requires 24.6 per cent N.

In addition to the above typical experiment, we have made a number of others in which the reaction and concentration of the acid was varied but without obtaining materially different results.

The yield of the methyl glyoxal derivative is small but it must be remembered that most of the alanine may be recovered unchanged, so that it is likely that the yield is relatively large compared with the amount of amino-acid decomposed.

The formation of methyl glyoxal from alanine necessitates the simultaneous liberation of ammonia and we have made a number of experiments which indicate that small amounts of ammonia are liberated from amino-acids with much greater ease than has been commonly supposed. We find, for example, that if a weak solution (2 per cent) of ordinary sodium phosphate is boiled in a distilling flask attached to a condenser until the distillate is perfectly free from ammonia when tested with Nessler's reagent and then a gram or so of an amino-acid, such as alanine, is added, the second distillate will be found to contain very definite traces of ammonia. On continuing the distillation, the amount of ammonia slowly diminishes but does not disappear entirely, and on allowing the previously boiled mixture to stand in the distilling flask for a short time (*e.g.*, 1 hour), a fresh formation of ammonia is apparent. Similar results were obtained on substituting sodium borate, prepared from boiled ammonia-free caustic soda and ignited boric acid, for the phosphate.

On adding a little freshly distilled ammonia-free acetic acid to a dilute alanine solution which has been well boiled with a little caustic soda to remove any ammonia present as an impurity and then digesting the mixture in the distillation apparatus at about 50° for an hour or two, we find that on making alkaline with caustic soda and redistilling, there is no difficulty in detecting ammonia in the distillate. Digestion of amino-acids with weak caustic soda solution ( $\frac{N}{100}$ ) also appears to lead to ammonia formation.

It need hardly be added that in all of the above experiments appropriate blank tests were constantly carried out and every effort made to guard against accidental contamination. We propose to study the reaction quantitatively.

#### *4. The formation of methyl glyoxal from glucose.*

Methyl glyoxal was shown by Pechmann to be somewhat volatile with steam and we made use of this property for its isolation from the complex mixture of substances resulting from the action of salts upon glucose.

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Glucose (50 grams) and sodium phosphate crystals (25 grams) were dissolved in water (500 cc.) and the mixture was distilled until about 300 cc. of distillate were obtained. An addition of 300 cc. of 5 per cent phosphate was then made and the distillation repeated until finally about 3 liters of distillate were obtained. The distillate gave a marked iodoform reaction and on treatment with *p*-nitrophenylhydrazine dissolved in acetic acid gave a red flocculent precipitate. The precipitate was collected and crystallized from either pyridine or better from nitrobenzene in deep crimson needles melting at 300°. The melting point was unchanged on mixing with pure methyl glyoxal dinitrophenylhydrazone.

ANALYSIS: 0.0882 gram dried at 150° gave 0.0214 gram N = 24.3 per cent N.  
 $C_{12}H_{14}O_4N_2$  requires 24.6 per cent N.

The yield of precipitate was small, about 0.5 gram, but no doubt only a very small proportion of the glyoxal formed was obtained in the distillate. Reference may be made here to the interesting experiments of Henderson<sup>8</sup> upon the loss of optical activity of glucose solutions on digestion with phosphates. The reaction undoubtedly deserves careful study. It is possible that the methyl glyoxal derivative we obtained is derived from acetol, but while this is doubtful it is not a matter of great importance for the purpose of the present experiments.

### 5. The formation of other $\alpha$ -ketonic aldehydes from $\alpha$ -hydroxy-acids and $\alpha$ -amino-acids.

We have been able to observe the formation, from a number of hydroxy- and amino-acids, of insoluble nitrophenylhydrazones giving reactions indicative of their being derived from  $\alpha$ -ketonic aldehydes; but in many cases we must defer an accurate description of the properties of the substances until we have had opportunity to study them more closely. The experiments were conducted in similar fashion to those already described with lactic acid and alanine, so that repetition will be unnecessary.

*Glycollic acid.* Glycollic acid (20 grams), *p*-nitrophenylhydrazine (2 grams) and water (200 cc.) were heated together, cooled, filtered and then digested at 39°. After a day a fine red granular

<sup>8</sup> This *Journal*, x, p. 3, 1911.



precipitate separated out which gave all the reactions of glyoxal dinitrophenylhydrazone. The quantity of precipitate increased steadily, but after a few days bright yellow crystals began to separate, which dissolved in caustic soda to give a bright red color. They proved to be the nitrophenylhydrazone of glyoxylic acid. The precipitate which first separated crystallized from nitrobenzene and toluene in small glistening deep red crystals melting with evolution of gas at  $302^{\circ}$ . On mixing the substance with glyoxal dinitrophenylhydrazone prepared from glyoxal, the melting point was unchanged. A trace of the substance on warming with caustic soda solution and a few drops of alcohol, gives a transitory greenish-blue color, passing to a deep blue and slowly changing to violet and finally brown red. The yield of pure substance was insufficient for analysis.

For comparison, glyoxal dinitrophenylhydrazone was prepared from glyoxal precisely as in the case of the methyl glyoxal derivative. The yield is practically theoretical. The substance has also been obtained by Wohl and Neuberg from glycollic aldehyde.<sup>9</sup>

The precipitate appearing during the later stages of the glycollic acid digestion was filtered off and washed with water and then dissolved in much boiling alcohol. On filtering, a small red precipitate consisting chiefly of glyoxal dinitrophenylhydrazone separated out. On concentrating the filtrate, yellow crystals of the nitrophenylhydrazone of glyoxylic acid were obtained. This substance has an indefinite melting point, beginning to decompose at a temperature somewhat above  $200^{\circ}$ , and on repeated crystallization, passes over into a less soluble modification, which is very sparingly soluble even in boiling nitrobenzene. The hydrazone dissolved in caustic soda to give a bright red color and was identical with the substance previously described by one of us, which was prepared directly from glyoxylic acid.<sup>10</sup>

**Glyceric acid.** The conversion of glyceric acid in 5 or 10 per cent solution on digestion with nitrophenylhydrazine (1 per cent) into the nitrophenylosazone of glyceric aldehyde is effected with remarkable ease. The osazone separates out in abundance after one to two hours and its quantity steadily increases as long as unchanged nitrophenylhydrazine is present.

<sup>9</sup> *Ber. d. deutsch. chem. Gesellsch.*, xxxiii, p. 3107, 1900.

<sup>10</sup> *This Journal*, iv, p. 235, 1908.

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The precipitate was filtered off and recrystallized from nitrobenzene and toluene. The osazone crystallizes in long thin scarlet needles melting at about 315° with evolution of much gas. It is sparingly soluble in alcohol, ether or amyl alcohol.

ANALYSIS: 0.1292 gram gave 0.0301 gram N = 23.3 per cent N.  
 $C_{12}H_{14}O_6N_4$  requires 23.8 per cent N.

A trace of the substance boiled with caustic soda and a little alcohol gives successively greenish-blue, deep blue, violet red and brown-red colors.

An attempt to prepare the above osazone from glyceric aldehyde, for purposes of comparison, gave a disappointing yield.

*Mandelic acid.* Experiments with mandelic acid similar to those with glycollic acid, gave a complicated mixture of substances. On washing the precipitate successively with hot sodium carbonate solution, water and alcohol, and then recrystallizing the residue from nitrobenzene, a small quantity of substance was obtained which resembled phenyl glyoxal dinitrophenylhydrazone in every respect. The amount of pure substance was insufficient for analysis.

The sodium carbonate washings on acidification gave a small quantity of phenyl glyoxylic acid nitrophenylhydrazone, crystallizing in hair-like needles melting at 163°–165°. Both of the above-mentioned hydrazones were prepared by independent methods for purposes of comparison.

Phenyl glyoxal dinitrophenylhydrazone was prepared from phenyl glyoxal (1 mol.) and nitrophenylhydrazine (2.2 mols.) dissolved in acetic acid (33 per cent). The red precipitate was washed with alcohol and recrystallized from nitrobenzene and toluene. It crystallizes in bright red needles melting at 302°–304°.

ANALYSIS: 0.1551 gram gave 0.3370 gram  $CO_2$  and 0.0559 gram  $H_2O$ .

	Found:	Calculated for $C_{20}H_{18}N_4O_6$ :
C .....	59.3	59.4
H .....	4.1	4.0

A trace of the substance warmed with caustic soda and alcohol gives successively carmine red, purple, clear light red fading finally to a light brown color.

Phenyl glyoxylic acid nitrophenylhydrazone was prepared by adding nitrophenylhydrazine dissolved in a slight excess of 5 per cent sulphuric acid to phenyl glyoxylic acid.<sup>11</sup> The hydrazone is precipitated as a yellow substance which crystallizes from weak alcohol, in which it is readily soluble, in hair-like needles melting at 163°–165°. It dissolves in caustic soda to give a bright red color.

ANALYSIS: 0.1056 gram substance gave 0.01568 gram N = 14.8 per cent N.  
 $C_{14}H_{11}N_3O_4$  requires 14.7 per cent N.

*Glycine.* The experiments with this substance were similar in every respect to those with alanine. There was no difficulty in detecting the formation of glyoxal dinitrophenylhydrazone, but the yield of precipitate was distinctly smaller than in the case of alanine.

*Aspartic acid.* On digesting a 1 per cent solution of aspartic acid with nitrophenylhydrazine at 39°, there is an abundant formation of a dinitrophenylhydrazone. The substance crystallizes from nitrobenzene and toluene in the form of small thick prisms, and on warming with caustic soda gives successively a greenish-blue, clear deep blue, followed by a more persistent violet-blue, finally turning reddish brown. The substance is apparently the dinitrophenylhydrazone of the  $\alpha$ -ketonic aldehyde corresponding to aspartic acid ( $COOH.CH_2.CO.CHO$ ). It will be studied further.

We have also obtained dinitrophenylhydrazones from other amino-acids including valine, leucine, phenylalanine, proline. These substances are sparingly soluble compounds with high melting points and give characteristic color reactions with caustic soda. We prefer to reserve their detailed description until we have had an opportunity of studying them more closely.

<sup>11</sup> A most convenient method for the preparation of phenylglyoxylic acid is as follows: Mandelic acid (10 grams) is neutralized with caustic potash and diluted to 500 cc. with ice and water. Potassium permanganate in 4 per cent solution (200 cc.) is added drop by drop to the cooled potassium mandelic solution, using a mechanical stirrer. Half an hour after all the permanganate has been added sulphur dioxide is passed in to dissolve the oxides of manganese. Sulphuric acid is then added in excess and the phenylglyoxylic acid extracted with ether. The yield is 90 per cent of the calculated one. (Cf. Evans: *loc. cit.*)

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### 6. *The fate of methyl glyoxal and phenyl glyoxal on perfusion through the liver. The formation of phenyl glyoxylic acid.*

The perfusions of dogs' livers with methyl and phenyl glyoxals were carried out as in the case of similar experiments reported from this laboratory,<sup>12</sup> with the exception that sodium phosphate was added to the perfusion mixture in order to provide for the prompt neutralization of any acid that might be formed.

*Methyl glyoxal.* The dog (14 kgm.) was starved for two days before operation. The liver was perfused for half an hour with a mixture containing blood, 500 cc.; phosphate, 500 cc. 5 per cent; methyl glyoxal, 3 grams; salt solution, 250 cc. After perfusion, the mixture was heated to coagulate protein and the liver was also cut up and boiled with water and the filtrates combined. The filtrates were evaporated almost to dryness, acidified with phosphoric acid and then taken up with gypsum. The dry powder was extracted with ether in the usual way. The ether extract was taken up in water and was found to be strongly laevo-rotatory. It was boiled with zinc carbonate and gave two crops of dextro-rotatory zinc lactate (4 grams). The rotations and analyses showed that both *d*- and *l*-lactic acids were present, the latter being in excess.

Crop I. (2.1 grams): 0.3302 gram dried at 120° lost 0.0585 gram H<sub>2</sub>O = 17.7 per cent.

ROTATION: 0.2667 gram air dried salt in 10 cc.; *l* = 2 dm.;  $\alpha = 0.1^\circ$ .  
 $[\alpha]_D = + 2.28^\circ$

Crop II. (1.9 grams): 0.2025 gram dried at 120° lost 0.0340 gram H<sub>2</sub>O = 16.8 per cent.

ROTATION: 0.1685 gram of dry salt in 10 cc.; *l* = 2 dm.;  $\alpha = 0.20^\circ$ .  
 $[\alpha]_D = + 5.93^\circ$

0.2702 gram of the mixed salts gave 0.0904 gram ZnO = 33.5 per cent ZnO.  
C<sub>6</sub>H<sub>5</sub>O<sub>6</sub> requires 33.4 per cent ZnO.

A second perfusion was made in which no methyl glyoxal was added. The dog (7 kgm.) had not been starved and the liver contained much glycogen. 500 cc. of 5 per cent phosphate solution were added to the blood, which after a perfusion lasting half an hour was analyzed as before. 1.7 grams of zinc lactate were obtained, all of which was derived from dextro lactic acid.

<sup>12</sup> This *Journal*, ix, p. 146, 1911.

ANALYSIS: 0.2516 gram dried at 120° lost 0.0335 gram  $H_2O$  = 13.2 per cent.

0.2150 gram gave 0.0714 gram  $ZnO$  = 33.2 per cent.

ROTATION: 0.2838 gram in 10 cc.;  $l$  = 2 dm.;  $\alpha$  =  $-0.42^\circ$ .

$[\alpha]_D = -8.53^\circ$ .

*Phenyl glyoxal.* Two experiments were made with phenyl glyoxal which were essentially similar to the methyl glyoxal experiment. In one experiment, 3 grams of phenyl glyoxal and 200 cc. of 5 per cent phosphate were added to the blood saline mixture, and perfusion carried on for one and three quarters hours. In the second experiment 4 grams of phenyl glyoxal and 400 cc. of phosphate were added and perfusion lasted one hour.

The aqueous filtrates from blood and liver were concentrated and, after acidifying with phosphoric acid, extracted with ether in a continuous extractor. The ethereal solution in each case was shaken twice with 10 cc. of saturated sodium bisulphite solution to separate any phenyl glyoxylic acid. The main ether extract was then evaporated to dryness and the crystalline residue of mandelic acid recrystallized from boiling toluene. The yield of mandelic acid varied from 1–1.6 grams. It was practically all the laevo-rotatory variety and melted at  $131^\circ$ .

ROTATION: 0.5776 gram in 20 cc.;  $l$  = 2 dm.;  $\alpha$  =  $-8.52^\circ$ .

$[\alpha]_D = -148^\circ$ .

The sodium bisulphite extracts were strongly acidified with sulphuric acid and extracted with ether. The ether residue, in addition to phenyl glyoxylic acid, contained much mandelic acid which apparently may be extracted from ether solutions by sodium bisulphite to a rather surprising extent.

The residue gave the benzene, thiophene, sulphuric acid test for phenyl glyoxylic acid in typical fashion.<sup>13</sup> The acid was further characterized as the nitrophenylhydrazone. The residue was dissolved in water, filtered and a clear solution of nitrophenylhydrazine in dilute sulphuric acid added. A bright yellow precipitate of phenyl glyoxylic acid *p*-nitrophenylhydrazone at once separated and was purified by recrystallization from dilute alcohol. In one experiment 0.2 gram was obtained, in the second 0.1 gram. The

<sup>13</sup> Some samples of technical benzene do not contain enough thiophene to give the reaction. It is therefore advisable to add thiophene separately.

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substance melted at 163°–165° and was identical with the product prepared directly from phenyl glyoxylic acid (section 5).

ANALYSIS: 0.1100 gram gave 0.0161 gram = 14.6 per cent N.  
 $C_{10}H_{11}N_2O_4$  requires 14.7 per cent N.

It may be noted here that on simple digestion of muscle tissue extracts with phenyl glyoxal, we have been able to detect readily the formation of phenyl glyoxylic acid.

### 7. The fate of methyl glyoxal and of l-lactic acid in the glycosuric organism.

For these experiments, we made use of dogs rendered glycosuric by daily injections of phlorhizin (1 gram) suspended in olive oil. The conditions of the experiments were similar to those of recently published experiments.<sup>14</sup>

*Methyl glyoxal.* A preliminary experiment was made in which 1.5 grams of methyl glyoxal in aqueous solution were given subcutaneously to a rabbit (1.5 kgm.) without effect, showing that it was relatively non-toxic. The methyl glyoxal used for the following experiment was freshly prepared by hydrolyzing the acetal according to Meisenheimer's method. It was given by stomach tube and produced no particular symptoms. The nitrophenylhydrazine test showed that no unchanged methyl glyoxal was excreted in the urine. The urine was collected in six-hour periods.

NITROGEN	GLUCOSE	G : N	ACETOACETIC ACID	SUBSTANCE GIVEN
3.67	14.40	3.92	0.012	9 gms. methyl glyoxal
<b>3.16</b>	<b>19.37</b>	<b>6.13</b>	<b>0.018</b>	
4.43	17.18	3.88	0.066	
		3.81		

The rise in G:N ratio on giving the methyl glyoxal is very marked. Using 3.87 as the average ratio, it is calculated that 9 grams of methyl glyoxal gave a little over 7 grams of "extra glucose."

In a second experiment in which the methyl glyoxal was given by subcutaneous injection, the G:N ratio rose from 3.7 to 7.66.

<sup>14</sup> This *Journal*, xiv, p. 321, 1913.

*l-Lactic acid.* The acid was prepared by resolving inactive lactic acid with morphine according to Irvine's<sup>15</sup> excellent method. The crystalline morphine *l*-lactate was decomposed by ammonia and the alkaloid filtered off. The ammonium lactate was converted into the calcium salt by prolonged boiling with lime. The pure crystallized calcium *l*-lactate was finally decomposed by heating with an equivalent weight of sodium sulphate and the calcium sulphate removed by filtration. The sodium lactate was given by stomach tube and evoked no symptoms.

NITROGEN	GLUCOSE	G: N	ACETOACETIC ACID	SUBSTANCE ADDED
3.74	14.21	3.81	0.155	12 gms. <i>l</i> -lactic acid as sodium salt.
<b>3.25</b>	<b>20.68</b>	<b>6.33</b>	<b>0.043</b>	
3.72	13.10	3.52	0.013	
		3.55		

Adopting 3.62 as the average G:N ratio, it is found that 12 grams of lactic acid furnished slightly over 9 grams of glucose.

<sup>15</sup> *Transactions of the Chem. Soc.*, lxxxix, p. 935, 1906.





## THE CHEMISTRY OF GLUCONEOGENESIS.

### THE RÔLE OF PYRUVIC ACID IN THE INTERMEDIARY METABOLISM OF ALANINE.

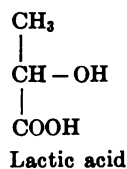
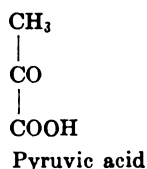
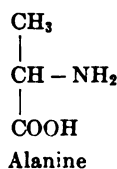
By A. I. RINGER.

WITH THE ASSISTANCE OF E. M. FRANKEL AND L. JONAS.

*the Department of Physiological Chemistry of the University of Pennsylvania, Philadelphia, Pa.)*<sup>1</sup>

(Received for publication, May 27, 1913.)

uvic acid occupies a singularly important position in intermediary metabolism. Because of its chemical relationship to pyruvic acid and lactic acid



was assumed to play a rôle in the intermediary metabolism of protein and carbohydrates.

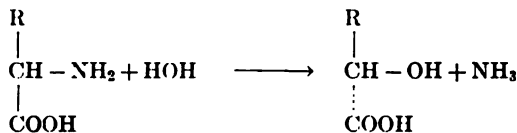
Until recent years was any attempt made to study the paths which the amino-acids undergo in the process of their catabolism. What was known was that the amino-acid broke down into a nitrogenous fraction that gave rise to urea and ammonia, and a carbon-free fraction which was "burnt."

With the development of our knowledge of the structural composition of the amino-acids and their related compounds, evidence began to accumulate which suggested definite reactions and definite paths of decomposition. Until the researches of Neubauer<sup>2</sup> came to light, it was the current belief that the  $\alpha$ -amino-acids underwent deamination in the animal body by a process of hydroly-

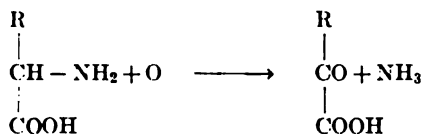
supported by a grant from the Rockefeller Institute for Medical Research.  
Neubauer: *Deutsch. Arch. f. klin. Med.*, xcv, p. 211, 1909.

## 146 Pyruvic Acid in Alanine Metabolism

ysis, whereby the  $\text{NH}_2$  was removed and an hydroxyl took its place.



Neubauer was the first to call attention to a different process of deaminization, *i.e.*, oxidative deaminization,

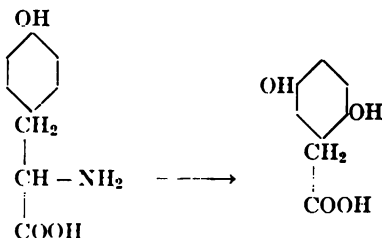


which has so much experimental evidence to support it that it is now almost universally accepted.

We shall not attempt to present a detailed account of the experiments which led to this conclusion, but will briefly state the facts that have a bearing on our present discourse.

Neubauer worked on a patient suffering from alkaptonuria. Such a patient presents an abnormality in his protein metabolism, which consists of his inability to burn tyrosine and phenylalanine and of the excretion of homogentisic acid in the urine. Neubauer and his associates utilized this fact in their study of the intermediary metabolism of tyrosine and phenylalanine<sup>3</sup> in the course of which they came to the following conclusions:

I. That tyrosine (para-oxyphenyl- $\alpha$ -amino-propionic acid) gives rise to extra homogentisic acid<sup>4</sup>



<sup>3</sup> O. Neubauer and W. Falta: *Zeitschr. f. physiol. Chem.*, xlii, p. 81, 1904.

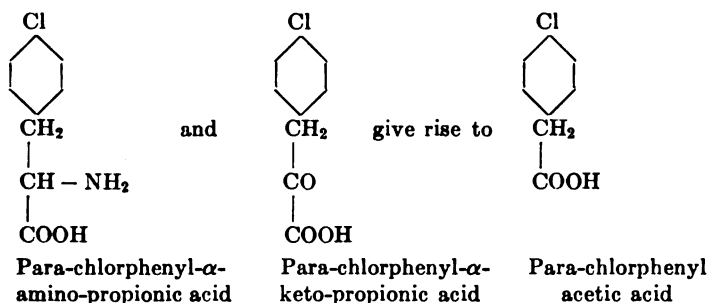
<sup>4</sup> Wolkow and Baumann: *ibid.*, xvi, p. 270, 1892.

II. That para-oxyphenyl- $\alpha$ -hydroxy-propionic acid,  $\text{HO.C}_6\text{H}_4\text{.CH}_2\text{.CHOH.COOH}$ , does not give rise to extra homogentisic acid.

III. That para-oxyphenyl- $\alpha$ -keto-propionic acid,  $\text{HO.C}_6\text{H}_4\text{CH}_2\text{.CO.COOH}$ , gives rise to extra homogentisic acid.

Since these  $\alpha$ -amino and  $\alpha$ -keto compounds can give rise to homogentisic acid, and the  $\alpha$ -hydroxy cannot, Neubauer concluded that the  $\alpha$ -amino compound cannot possibly give rise to the  $\alpha$ -hydroxy acid as an intermediary body. He then conceived of the "oxidative deamination" theory, which seems to explain his findings beautifully.

Additional support to this theory was rendered by Friedmann and Masse<sup>5</sup> who showed that



whereas para-chlorophenyl- $\alpha$ -hydroxy-propionic acid,  $\text{Cl.C}_6\text{H}_4\text{.CH}_2\text{.CHOH.COOH}$ , did not give rise to para-chlorophenyl acetic acid. This again showed that the hydroxy acid could not possibly have been an intermediary compound in the metabolism of the amino-acid.

On the strength of his findings Neubauer was led to extend his theory to the entire series of  $\alpha$ -amino-acids and suggested that alanine, in the animal body, gives rise to pyruvic acid, which may secondarily give rise to lactic acid.

Reviewing the evidence in support of this theory, one feels convinced of the soundness of the conclusion in the case of the aromatic compounds. With regard to the open chain  $\alpha$ -amino-acids, however, there seems to be little direct evidence. It is true that the conversion of  $\alpha$ -keto-acids into alanine by the ani-

<sup>5</sup> Friedmann and Masse: *Biochem. Zeitschr.*, xxvii, p. 97, 1910.

mal organism has been proven and that ketonic acids may in the animal body go over with great ease into the corresponding hydroxy acids, which process has been shown repeatedly to be reversible; still we feel that *it is not proven satisfactorily that alanine in its catabolism must pass through pyruvic acid, and that lactic acid can arise only secondarily from pyruvic acid.*

We raise this question because in experiments which we have performed with the object of ascertaining the degree to which pyruvic acid can give rise to extra glucose in diabetic animals, it was found that pyruvic acid does not behave in the way it would if it really occupied a definite and obligatory place in the intermediary metabolism between alanine and lactic acid.

During the course of our work we have come across several substances whose gluconeogenetic properties are not constant, which have the power of yielding more glucose at one time and less at other times, and we have been inclined to attribute their gluconeogenetic properties to certain factors of equilibrium, which turn the reaction of their metabolism in one path at one time and in another path at another time. According to our experience pyruvic acid may be classed among this group.

The status of pyruvic acid in metabolism has been the subject of very considerable discussion during the past year. Parnas and Baer<sup>6</sup> perfused the liver of the tortoise with Ringer's solution, blood and oxygen to which 4 grams of pyruvic acid as sodium salt had been added, and found no increase in the glycogen. On the basis of this one experiment they drew the conclusion that pyruvic acid cannot be classed among the glucogenetic substances. In our estimation, this conclusion is not at all justified.

P. Mayer<sup>7</sup> administered 7 to 8 grams of pyruvic acid to normal rabbits and found that they developed glucosuria, the severity of which depended upon the state of nutrition of the animal. In one rabbit he obtained 2.4 grams of glucose in the twenty-four hours following the administration of pyruvic acid. In animals which had fasted for periods of ten to eleven days, the administration of pyruvic acid was not followed by glucosuria, but by an increase of the glucose concentration of the blood and by an increase in the glycogen in the liver. In this communication Mayer left open the question of the origin of the glucose. In a second paper

<sup>6</sup> Parnas and Baer: *Biochem. Zeitschr.*, xli, p. 386, 1912.

<sup>7</sup> Paul Mayer: *ibid.*, xl, p. 441, 1912.

on the subject<sup>8</sup> (which appeared after our work was far advanced) Mayer studied the influence of pyruvic acid on gluconeogenesis in phlorhizinized dogs and rabbits. He found that pyruvic acid did not give rise to any extra glucose, and that in some cases the urinary constituents were greatly diminished after the pyruvic acid administration. In experiment X the glucose elimination dropped from 13.48 grams in the fore period to 1.45 grams (!) in the experimental period, and the nitrogen dropped from 2.92 grams to 0.53 gram (!). In experiment XI the glucose elimination dropped from 32.93 grams to 3.41 grams (!). The kidneys of these animals were examined microscopically and extensive tubular degeneration was found. Mayer concluded that pyruvic acid is a toxic substance which causes a disturbance in the sugar and nitrogen elimination and which acts by decreasing the permeability of the kidneys.

To all of these conclusions we object most emphatically. We gave Kahlbaum's pyruvic acid seven times to six different dogs in quantities varying from 8.8 to 13.2 grams, administered subcutaneously and orally, and never have we obtained any of the toxic symptoms described by Mayer. In no case did we get the peculiar drop in the glucose and nitrogen eliminations, and in no case did we observe any sign or symptom of any kidney involvement. We have, however, seen a picture of Mayer's experiences with pyruvic acid after subcutaneous and, under certain circumstances also, after oral administration of tartaric acid.<sup>9</sup> The resemblance is so close that we do not hesitate for a moment to attribute Mayer's results to a contamination of his pyruvic acid with tartaric acid. This is all the more probable since pyruvic acid is very largely prepared by the distillation of tartaric acid.

In all of our experiments pyruvic acid appears to be a glucose-yielding substance, the question is only one of degree. The methods employed in these experiments are the same as those employed and described in the previous papers of this series.

In experiment XXII period VIII, 10 grams of pyruvic acid as sodium salt were administered subcutaneously. The glucose elimination rose from 33.6 grams in the fore period to 40.91 grams in the experimental period, and returned to 35.75 and 32.54 grams in

<sup>8</sup> Paul Mayer: *ibid.*, xlix, p. 486, 1913.

<sup>9</sup> Underhill: this *Journal*, xii, p. 115, 1912. We have been able to corroborate Underhill's findings. Our results will be published soon.

the after periods IX and X respectively. The amount of extra glucose eliminated was 8.21 grams.

In experiment XXIII period III, 10 grams of pyruvic acid as sodium salt were given subcutaneously. The glucose elimination rose from 28.54 to 32.04 grams and returned to 22.84 grams in the after period. The D:N ratio, which was 3.44 in the fore period, rose to 4.18 and came down to 3.6 in the after period. The amount of extra glucose was 5.09 grams.

In experiment XXIV period III, 13.2 grams of pyruvic acid as sodium salt were given subcutaneously. The glucose elimination rose from 17.58 grams to 19.18 grams. The amount of extra glucose eliminated was 2.25 grams.

In experiment XXV period IV, 8.8 grams of pyruvic acid as sodium salt were given subcutaneously. The amount of extra glucose eliminated was 5.1 grams.

In experiment XXVI period V, 8.8 grams of pyruvic acid as sodium salt were given subcutaneously. The amount of extra glucose eliminated was 1.16 grams.

In two other experiments, 10 grams of pyruvic acid as sodium salt were given *per os*. The dog vomited part of the ingested material each time. The animals lived for a long time afterwards and no change in the urinary constituents was noticed. The experiments are not reported in detail because the vomitus contaminated part of the urine.

From all these experiments we see very clearly that pyruvic acid cannot be considered a toxic substance (in Mayer's sense) and that in most of the cases pyruvic acid yields large quantities of extra glucose.

However, when we come to compare the results obtained after pyruvic acid administration with those obtained after alanine<sup>10</sup> and lactic acid,<sup>11</sup> we note a very marked difference. While alanine and lactic acid never fail to yield large quantities of extra glucose, pyruvic acid at times yields very small quantities (experiments XXIV and XXVI). This fact makes it very certain that *in the process of catabolism alanine cannot have pyruvic acid as its principal product of intermediary metabolism, and that alanine does not undergo oxidative deaminization*. Alanine and lactic acid yield glucose in quantities so similar to each other, that one seems

<sup>10</sup> Ringer and Lusk: *Zeitschr. f. physiol. Chem.*, lxvi, p. 106, 1910.

<sup>11</sup> Mandel and Lusk: *Amer. Journ. of Physiol.*, xvi, p. 129, 1906.

justified in concluding that the conversion of the former into the latter is quantitative.

In a previous communication<sup>12</sup> it was shown that malic acid yields glucose in quantities similar to aspartic acid, and it was suggested that the former was an intermediary product in the metabolism of the latter. It becomes of interest to know what rôle, if any, the corresponding ketone—oxalacetic acid—plays in it. These experiments are in progress and will be reported soon.

EXPERIMENT XXII. *Twelve-hour periods.*

DATE 1913	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL GLUCOSE	D : N	EXTRA GLUCOSE	NH <sub>4</sub> N	ACETONE AND ACETO-ACETIC ACID	REMARKS
Feb.									
2	IV		9.82	38.38	3.91		0.89	1.25	
3	V		9.12	33.60	3.68		0.88	1.04	
3	VI	17.40	8.04						
4	VII		8.00						
4	VIII	17.00	8.65	40.91	4.73	8.21	0.505	0.308	10 gms. pyruvic acid as Na salt given subcutaneously.
5	IX		9.18	35.75	3.89		0.50	0.279	
5	X	16.64	8.62	32.54	3.77		0.49	0.283	

EXPERIMENT XXIII. *Twelve-hour periods.*

3	II	12.40	8.28	28.54	3.44			0.340	
3	III		7.66	32.04	4.18	5.09		0.160	10 gms. pyruvic acid as above.
4	IV		6.34	22.84	3.60			0.430	
4	V	11.54	6.13	23.38	3.81			0.501	
5	VI		5.81	20.51	3.52			0.840	
5	VII	11.20	5.32	20.08	3.77			0.514	
6	VIII		5.21	18.40	3.53				

<sup>12</sup> Ringer, Frankel and Jonas: this *Journal*, xiv, p. 539, 1913.

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EXPERIMENT XXIV. *Twelve-hour periods.*

DATE 1913	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL GLUCOSE	D:N	EXTRA GLUCOSE	NH <sub>4</sub> N	ACETONE AND ACETO- ACETIC ACID	$\beta$ -OXY BUTYRIC ACID	REMARKS
Apr. 30	I		4.77	18.45	3.87		0.27	0.07	0.24	
May 1	II		5.58	17.58	3.16		0.36	0.11	0.47	
1	III	8.00	5.26	19.18	3.65	2.25	0.36	0.13	0.39	13.2 gms. py- ruvic acid as above
2	IV		5.74	18.75	3.27		0.29	0.10	0.40	
2	V	7.98	4.47	15.00	3.36		0.19	0.13	0.49	

EXPERIMENT XXV. *Twelve-hour periods.*

May										
1	I	9.31	3.77	12.63	3.35			0.22	1.21	
2	II		3.85	12.10	3.14		0.41	0.39	2.14	
2	III	9.11								
3	IV		3.34	13.80	4.14	5.10	0.23	0.18	0.69	8.8 gms. py- ruvic acid as above.
3	V	8.94	3.30	11.60	3.52		0.23	0.35	1.82	
4	VI		4.40	13.10	2.98		0.35	0.60	2.56	

EXPERIMENT XXVI. *Twelve-hour periods.*

May										
16	IV		7.80	28.60	3.68			0.13	0.35	
16	V	12.44	8.35	32.30	3.87	1.16		0.09	0.34	8.8 gms. py- ruvic acid as above.
17	VI		7.82	29.51	3.78			0.22	1.00	

SUMMARY.

I. Experiments on phlorhizinized dogs have shown that pyruvic acid is capable of yielding extra glucose in the diabetic organism.

II. In some cases the quantity of glucose was found to be much less than arises from similar amounts of alanine and lactic acid.

III. It is concluded that pyruvic acid cannot be considered a necessary intermediary product in the conversion of alanine into lactic acid and that alanine cannot be considered to undergo oxidative deamination.



## SPHINGOMYELIN.

### FIRST PAPER.

#### ON THE PRESENCE OF LIGNOCERIC ACID AMONG THE PRODUCTS OF HYDROLYSIS OF SPHINGOMYELIN.

By P. A. LEVENE.

(From the Rockefeller Institute for Medical Research, New York.)

(Received for publication, May 28, 1913.)

The products of hydrolysis of sphingomyelin have not been the subject of a careful investigation in recent years. The knowledge of the acid components of the substance is limited to the statement of Thudichum<sup>1</sup> that the principal acid taking part in the formation of the molecule is a saturated fatty acid, of the composition  $C_{18}H_{36}O_2$ , melting at  $59^{\circ}C.$ , and hence isomeric with stearic acid. In course of the present investigation it was found that the acid had the composition  $C_{24}H_{48}O_2$ , a melting point of  $81^{\circ}C.$ , and formed an ethyl ester melting at  $55-56^{\circ}C.$ , and hence was lignoceric acid. A sample of the ester mixed with that of an ester of lignoceric acid showed the same melting point. The molecular weight of the acid was 368.

### EXPERIMENTAL PART.

Sphingomyelin in lots of 40 grams was taken up in 400 cc. of alcohol containing 7 per cent of sulphuric acid and heated with reflux condenser on the water bath for six hours. The product of hydrolysis was allowed to remain at room temperature ( $20^{\circ}C.$ ) over night, and the ester separated in form of beautiful scales. These were filtered, again taken up in alcohol containing 7 per cent of sulphuric acid, and heated as before for an additional five hours. The ester which separated on the second esterification was filtered off with suction, and recrystallized out of acetone at room

<sup>1</sup> *A Treatise on the Chemical Constitution of the Brain*, London, 1884.

## 154 Lignoceric Acid from Sphingomyelin

temperature. Filtered and dried in a vacuum desiccator it had a sharp melting point of 55–56°C. Mixed with a sample of an ester of lignoceric acid obtained on oxidation of cerebronic acid it showed the same melting point of 55–56°C.

0.1428 gram of the substance gave on combustion after Dennstedt 0.4110 gram of CO<sub>2</sub> and 0.1686 gram of H<sub>2</sub>O.

	Calculated for C <sub>24</sub> H <sub>48</sub> COOC <sub>2</sub> H <sub>5</sub> :	Found:
C.....	78.80	78.50
H.....	13.10	13.20

For estimation of the molecular weight the ester was saponified by means of alcohol containing an  $\frac{N}{2}$  solution of sodium hydrate.

1.3491 grams of the substance neutralized on boiling for four hours 3.57 cc. of  $\frac{N}{2}$  alkali. M. W. = 395.

1.3734 grams of the substance neutralized under the same conditions 3.50 cc. of  $\frac{N}{2}$  alkali. M. W. = 391.

1.7848 grams of the substance neutralized 4.55 cc. of alkali. M. W. = 391.

	Calculated for C <sub>24</sub> H <sub>48</sub> O <sub>2</sub> :	Found:		
		I	II	III
M. W.....	368	367	368	363

In order to obtain the free acid the ester was saponified by means of alcoholic sodium hydrate. The soap was decomposed by means of hydrochloric acid, and the free acid repeatedly taken up in water containing hydrochloric acid and warmed on the water bath until the acid melted to an oil. The free acid was finally transformed into the lead salt, and the lead salt decomposed by means of sulphurated hydrogen. The free acid was recrystallized out of toluene. It melted sharply at 81°C., and had the following composition.

0.1160 gram of the substance gave on combustion after Dennstedt 0.3331 gram of CO<sub>2</sub> and 0.1366 gram of H<sub>2</sub>O.

	Calculated for C <sub>24</sub> H <sub>48</sub> O <sub>2</sub> :	Found:
C.....	78.30	78.33
H.....	13.00	13.03

## ON CHONDROITIN SULPHURIC ACID.

SECOND PAPER.

BY P. A. LEVENE AND F. B. LA FORGE.

(From the Rockefeller Institute for Medical Research, New York.)

(Received for publication, May 29, 1913.)

It was stated in the previous communication<sup>1</sup> on this subject that chondrosin is composed of two substances, both in some way related to carbohydrates. One was identified as *d*-glucuronic acid; regarding the nature of the other component the views of different writers were most contradictory. Thus, Schmiedeberg<sup>2</sup> was led to the belief that the substance was glucosamine and Orgler and Neuberg<sup>3</sup> announced it to be monoamino-tetrahydroxycaproic acid. Indeed these authors have described crystalline salts of the acid, which were supposedly obtained from the products of barium hydrate hydrolysis of the complex. The most recent reference to the nature of the substance was made by Fränkel,<sup>4</sup> who regarded it as an amino-glucuronic acid.

Some of the properties of the chondrosin seemed to justify the view of Orgler and Neuberg. Namely, the substance displayed a marked resistance towards boiling mineral acids; and, on the other hand, was readily hydrolyzed by alkalies. For this reason an attempt was made to repeat the experiment of Orgler and Neuberg. Soon, however, it became evident that success could not be expected from these experiments for the reason that even at the low temperature of the thermostat room (40°C.) an aqueous solution of barium hydrate caused chondrosin to give up all its nitrogen in form of ammonia. At this stage of the investigation it was concluded to search for some indirect evidence re-

<sup>1</sup> This *Journal*, xv, p. 69.

<sup>2</sup> Schmiedeberg: *Arch. f. exp. Path. u. Pharm.*, xxviii, p. 358, 1891.

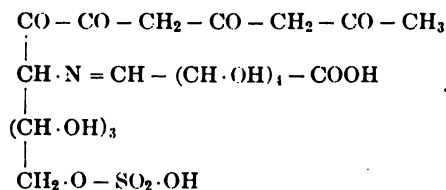
<sup>3</sup> Orgler and Neuberg: *Zeitschr. f. physiol. Chem.*, xxxvii, p. 407, 1903.

<sup>4</sup> S. Fränkel: *Ann. d. Chem.*, cccli, p. 344, 1907.

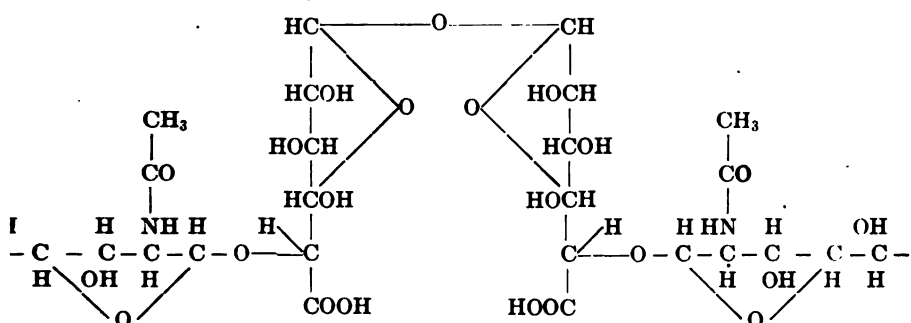
garding the nature of the substance, and for this reason an attempt was made to obtain laevulinic acid on treatment of chondroitin sulphuric acid with strong mineral acids. It was shown by Neuberger that glucosamine under certain conditions yields laevulinic acid particularly after preceding deaminization with nitrous acid. Chondrosin was therefore deaminized by means of sodium nitrite and hydrochloric acid, and the product boiled with mineral acids. Two experiments were performed and each yielded small quantities of laevulinic acid. This observation encouraged a renewal of the efforts to obtain glucosamine on direct treatment of chondroitin sulphuric acid with mineral acid. Similar to other workers we met with failures when the experiments were performed on the crude acid sodium salt of chondroitin sulphuric acid. The experiment reported in this communication was performed on a barium salt of chondroitin sulphuric acid free from any mineral impurities. The result of the experiment was most gratifying, as glucosamine hydrochloride was obtained, without an effort, in analytically pure condition.

Thus, for the first time all the components of chondroitin sulphuric acid were isolated and identified. They were those assumed on indirect evidence by Schmiedeberg, namely, sulphuric acid, acetic acid, glucosamine and glucuronic acid.

However, the results of the present investigation necessitate a revision of Schmiedeberg's view regarding the chemical structure of the complex molecule. According to Schmiedeberg's view the structure of the substance is presented by the following graphic formula:



The results of the present investigation lead to the following graphic expression of its composition:



The two views differ in the following. The theory of Schmiedeberg assumes the union of the glucosamine and of glucuronic acid through the formation of a nitrogen bridge. This view is erroneous for the reason that in chondrosin the nitrogen is present in the form of a free amino group, and hence can be removed by nitrous acid. On the basis of the present work one may assume a glucosidic union between the glucosamine and one of the secondary alcohol groups of the glucuronic acid. Of these the  $\gamma$ -position is excluded by virtue of the  $\gamma$ -oxidic structure of glucuronic acid.

The second difference of the two theories relates to the number and place of the acetyl groups entering in the structure of the molecule. Schmiedeberg assumed the presence of three acetyl groups in chondroitin, basing his view exclusively on the percentage composition of the amorphous salts of the substance.

In the course of the present work it was not possible to detect more than one acetyl group on hydrolysis of chondroitin sulphuric acid with mineral acids, or with an aqueous solution of barium hydrate. The presence of only one acetyl group was suggestive on the basis of analytical data, showing in chondroitin sulphuric acid a ratio of N:C = 1:14. The same ratio was found by Levene and Jacobs<sup>5</sup> on the analysis of the barium salt of glycothionic acid obtained from tendo-mucin.

The place of the acetyl group is made obvious by the fact that the nitrogen of chondroitin sulphuric acid is not removed in form of nitrogen gas by nitrous acid, and it was stated already that

<sup>5</sup> Levene and Jacobs: *Journ. of Exp. Med.*, x, p. 557, 1908.

chondrosin contains its nitrogen in form of an unsubstituted amino group.

Finally, the theory expressed here assumes a glucosidic union of two chondrosin molecules, for the reason that this view offers the simplest explanation to the observation that chondroitin does not possess reducing power for Fehling's solution, while chondrosin does.

#### EXPERIMENTAL.

##### *Laevulinic acid from chondrosin.*

Fifty grams of chondroitin sulphuric acid barium salt were converted into chondrosin. The solution deaminized with sodium nitrite and evaporated to about 75 cc. in vacuum. Two volumes of 40 per cent hydrochloric acid were then added and the solution boiled for twenty hours. It was then filtered from melanin and extracted several times with ether. The ethereal extract was dried with sodium sulphate, evaporated to dryness and the residue taken up in 250 cc. of water. It contained tin which was removed with hydrogen sulphide and the filtrate, after having been evaporated to remove the latter, was again extracted with ether. The residue from the ethereal solution was taken up in about 2 cc. of water nearly neutralized with ammonia, and the chlorine was removed with a few drops of silver nitrate solution. The addition of concentrated silver nitrate and ammonia to the filtrate produced a white precipitate which was washed with alcohol and ether and dried. The yield was 0.2 gram.

0.1260 gram substance gave 0.0602 gram Ag.

	Calculated for	
	$C_6H_7O_6Ag$ :	Found:
Ag .....	48.24	48.57

##### *Glucosamine hydrochloride from chondrosin.*

Fifty grams of chondroitin sulphuric acid barium salt were converted into chondrosin, the solution evaporated to a syrup in vacuum and taken up into 150 cc. of water. 150 cc. of concentrated hydrochloric acid were then added together with 10 grams of stannous chloride, and the solution boiled for six hours under a reflux condenser. Without filtering from the separated melanin, the product was diluted to 500 cc. and the tin removed with hydro-

gen sulphide. The colorless filtrate from the tin sulphides was concentrated in vacuum to a thick syrup which crystallized spontaneously to a solid cake. This was ground with 95 per cent alcohol in a mortar, filtered and washed repeatedly with alcohol and finally with ether and dried in vacuum. The yield was 9 grams; 2 grams crystallized from the alcoholic mother liquors upon standing.

0.1481 gram of substance gave 17.7 cc. amino nitrogen at 20°, 766 mm.

0.1226 gram substance from alcoholic mother liquors washed and dried in vacuum gave 0.1512 gram CO<sub>2</sub> and 0.0750 gram H<sub>2</sub>O.

0.1608 gram of substance recrystallized from 80 per cent alcohol with addition of a few drops of concentrated HCl required 7.72 cc. AgNO<sub>3</sub> solution (1 cc. = 0.00352 Cl).

	Calculated for C <sub>8</sub> H <sub>10</sub> O <sub>4</sub> N HCl:	Found:
N .....	6.51	6.87
Cl .....	16.45	16.90
C .....	33.40	33.63
H .....	6.54	6.79

0.1001 gram substance in 10 cc. H<sub>2</sub>O rotated in a 1 dm. tube with D-light + 1°.

$$[\alpha]_{20}^D = 99.9.$$

*Nitrogen-carbon ratio in chondroitin sulphuric acid barium salt.*

0.5346 gram of barium salt dried to constant weight in vacuum at 100° gave 8.25 cc. of  $\frac{N}{16}$  NH<sub>3</sub>.

0.2197 gram of barium salt gave 0.0680 gram H<sub>2</sub>O and 0.2110 gram CO<sub>2</sub>.

N .....	2.18 per cent.
H .....	4.45 per cent.
C .....	26.18 per cent.

$$N:C = 1:14.1$$

*Acetic acid determinations.*

Twenty-five grams of the barium salt of chondroitin sulphuric acid were re-purified by dissolving in 2 liters of distilled water with the addition of 10 grams of pure barium chloride and precipitating by the addition of 1 liter of 95 per cent alcohol. The precipitate was washed chlorine-free with 50 per cent alcohol and finally with absolute alcohol and ether, and dried in vacuum. About 10 grams of this material were dissolved in 10 cc. of water.

The acetic acid determinations were carried out in the following manner. The solution of the chondroitin sulphuric acid barium salt was subjected to simultaneous hydrolysis with 25 per cent sulphuric acid and distillation at ordinary pressure. The distillate was collected in a receiver, cooled with ice and protected from the atmospheric carbon dioxide by soda lime, while the volume of the solution in the reaction flask was kept constant by the addition of water through a dropping funnel.

5 cc. solution gave 7.40 cc.  $\frac{N}{10}$   $\text{NH}_3$  (Kjeldahl).

5 cc. solution gave 7.60 cc.  $\frac{N}{10}$  acetic acid.

5 cc. solution gave 7.80 cc.  $\frac{N}{10}$  acetic acid.

5 cc. solution gave 7.55 cc.  $\frac{N}{10}$  acetic acid.

Five cubic centimeters of another solution, which corresponded to 8.50 cc.  $\frac{N}{10}$   $\text{NH}_3$  (Kjeldahl) were hydrolyzed in the same apparatus with 20 per cent barium hydrate until no more ammonia was given off. The solution was then acidified with sulphuric acid and the acetic acid determination carried out as above.

5 cc. solution gave 9.75 cc.  $\frac{N}{10}$  acetic acid.



## STUDIES ON COTTON SEED MEAL TOXICITY. II.

### IRON AS AN ANTIDOTE.

By W. A. WITHERS AND J. F. BREWSTER.

WITH THE COLLABORATION OF R. S. CURTIS, G. A. ROBERTS, L. F. WILLIAMS AND J. W. NOWELL.

(From the North Carolina Agricultural Experiment Station, Raleigh.)

(Received for publication, May 31, 1913.)

It is a well established fact that cotton seed meal will produce death when fed for long periods and in large amounts to swine and smaller animals, and that care must be exercised even when feeding it too freely to cattle. This experiment station has been investigating this subject for some time and has made publication<sup>1</sup> of some of the previous work.

In the course of our experiments we were led to the hypothesis that the toxic principle<sup>2</sup> of cotton seed meal was a constituent group of the protein molecule containing loosely bound sulphur, and that the toxic effect of the meal was due to the action of this group upon the iron of the blood.

We reserve for a subsequent paper our experiments already performed and in process as they bear upon this hypothesis, but we desire in this paper to present the results of our experiments as they bear upon the efficiency of iron as an antidote for cotton seed meal poisoning.

In order to ascertain the toxicity of cotton seed meal under the conditions existing in our laboratory we fed meal to several rabbits until each died. Molasses was fed with the meal to increase the palatability. We began this feeding on April 23, 1911.

<sup>1</sup> *Science*, xxxvi, pp. 31-32, 1912; *Proceedings of the Society for the Promotion of Agricultural Science*, 1912, pp. 19-21; this *Journal*, xiv, pp. 53-58, 1913; Report of the North Carolina Agricultural Experiment Station, 1911-1912, pp. 141-149.

<sup>2</sup> This was announced in a paper read before the North Carolina Academy of Science, April 26, 1913, at its meeting in Greensboro, N. C.

Fifteen grams of cotton seed meal were given as the daily feed for each rabbit, which was approximately 10 grams per kilogram. The amount refused was weighed and deducted from the whole. Meal was fed at night and green feed in the morning. The feeding was carried on in galvanized iron cages. All in all we have fed cotton seed meal to twenty-two rabbits the average initial weight of which was a little over 1.5 kgms. As a rule the rabbits ate the meal well during the first few days and made gains in weight. But towards the end they began to refuse the meal in whole or in part and soon thereafter died. One rabbit died as early as six days after beginning to eat the meal, one survived for twenty-two days, but none beyond that time. On an average death ensued on the thirteenth day. Every rabbit lost in weight, the average loss for the period being about one-fifth of the initial weight of the animal. The average consumption of meal for each rabbit was 10 grams daily, or a total of about one-twelfth of the average initial weight of the rabbits.

The record of each rabbit is shown in the following table.<sup>3</sup>

*Table showing toxicity of cotton seed meal to rabbits.*

NUMBER OF RABBIT	WEIGHT OF RABBIT			COTTON SEED MEAL CON- SUMED	NO. OF DAYS SURVIVED FEEDING
	Initial	Final	Loss		
500	975	850	125	105	8
501	1225	1085	140	183	13
502	1130	1030	100	132	13
503	1320	1077	243	225	16
504	970	795	175	84	8
505	1370	1120	250	108	9
523	1580	1050	530	219	16
524	2370	1600	770	150	14
525	1670	1200	470	162	21
534	2560	1768	792	218	16
535	1940	1360	580	137	13
538	1605	1230	375	158	12
598	1154	1100	54	75	7
600	1307	1122	185	77	11
601	1591	1085	506	48	14
602	1260	1212	48	60	6
603	1260	912	348	71	16

<sup>3</sup> All weights in this paper refer to grams.

*Table showing toxicity of cotton seed meal to rabbits—Continued.*

NUMBER OF RABBIT	WEIGHT OF RABBIT			COTTON SEED MEAL CONSUMED	NO. OF DAYS SURVIVED FEEDING
	Initial	Final	Loss		
681	2440	2082	358	209	22
745	2000	1200	800	92	14
746	1700	1350	350	131	13
850	1640	1430	210	143	13
851	1630	1570	60	132	11
Average.	1577.1	1237.6	339.5	132.7	13

*Iron as an antidote for toxicity of cotton seed meal.*

During our experiments we were feeding to some rabbits the residue left after treating cotton seed meal in the cold with an aqueous solution of sodium carbonate (3.8 grams dissolved in 286 cc. of water to 90 grams of meal, the treatment being for twenty-four hours). Each rabbit was getting the equivalent of 15 grams of whole meal. On the fourteenth day of the feeding two of the rabbits were dead, one was sick and all had lost in weight. From this we inferred that the feed was toxic. To test the effect of iron as an antidote to the poisonous effect of the feed, we began on the fourteenth day to supply in addition to this feed a solution of citrate of iron and ammonia (0.7 gram in 10 cc. of water at first each day and later on alternate days). This addition of iron to the feed was continued for fourteen days. The sick rabbit consumed the iron at once and appeared to be well the next day. From the time he was given iron he ate all the meal supplied to him except on the first day, when he refused part of it. The rabbits which had not been sick never refused any meal during the period after they began to receive iron. All three of them gained in weight during the fourteen-day period in which iron was given with the meal fraction whereas during the previous fourteen-day period in which the same fraction was fed without iron they had lost in weight. During the iron-feeding period each rabbit remained well, but during the previous feeding period without iron two had died and one was made sick. The contrast between the two feeding periods and the detailed records for each rabbit are shown in the following table.

## Toxicity of Cotton Seed Meal

*Table showing the effect of feeding a cotton seed meal residue without iron.*

RABBIT NUMBER	WEIGHT OF RABBIT			COTTON SEED MEAL CONSUMED	DAYS FED	CONDITION OF RABBIT AT END OF PERIOD
	Initial	14th day	Loss			
844	1430	1220	210	195	14	Died 14th day.
845	1800	1600	200	150	14	
846	1750	1620	130	165	14	Sick 14th day.
847	1760	1680	80	192	14	
848	1520	1400	120	150	11	Died 11th day.
Average.	1652	1504	148	170		

*Table showing the results of feeding iron with the cotton seed meal fraction for fourteen days.*

RABBIT NUMBER	WEIGHT OF RABBIT			COTTON SEED MEAL CONSUMED	IRON SALT CONSUMED	DAYS FED	CONDITION OF RABBIT AT END OF PERIOD
	14th day	28th day	Gain				
845	1600	1710	110	210	7.7	14	Alive and normal.
846	1620	1670	50	203	7.7	14	Alive and normal.
847	1680	1700	20	210	7.7	14	Alive and normal.
Average.	1633	1693	60	208	7.7	14	

This feed was discontinued fourteen days after beginning the administration of iron. At that time the rabbits were apparently perfectly healthy.

This experiment indicates that iron may overcome the toxic effects of cotton seed meal where they have appeared, and if fed beforehand may prevent their appearance.

*Effect of feeding iron with whole cotton seed meal.*

As early as we could secure them we began feeding 15 grams of cotton seed meal daily to each of six rabbits, and along with the daily feed we gave 0.3 gram of citrate of iron and ammonia. For four days previous to the meal feeding we began to supply the iron solution. The detail is shown in the following table.

RABBIT NUMBER	WEIGHT OF RABBIT			FEED CONSUMED		DAYS FED	CONDITION OF RABBIT AT END OF PERIOD
	Initial	23d day	Gain	Cotton seed meal	Iron salt		
870	1370	1655	285	345	8.1	23	Alive and normal.
871	1550	1700	150	345	8.1	23	Alive and normal.
872	1420	1625	205	345	8.1	23	Alive and normal.
873	1330	1550	220	345	8.1	23	Alive and normal.
874	1110	1405	295	345	8.1	23	Alive and normal.
875	1500	1630	130	345	8.1	23	Alive and normal.
Average.	1380	1594	214	345	8.1	23	

It will be seen that each rabbit ate all the cotton seed meal and all the iron salt supplied and each gained in weight. By referring to the table at the beginning of this paper it will be seen that each of these six rabbits ate more cotton seed meal when iron was fed with the meal than any of the twenty-two rabbits could eat without the addition of iron. It will be noticed also by referring to the same table that every one of the twenty-two rabbits was killed by cotton seed meal before the twenty-third day if iron was not added, whereas all the six animals receiving iron along with the cotton seed meal are alive on the twenty-third day, are gaining in weight and are normal so far as we can judge.<sup>4</sup> This experiment would indicate the great efficiency of iron as an antidote for cotton seed meal poisoning.

Confirming these results with six rabbits we have results obtained by feeding to a pair of rabbits a solution of citrate of iron and ammonia with whole cotton seed meal.

RABBIT NUMBER	WEIGHT OF RABBIT			FEED CONSUMED		DAYS FED	CONDITION OF RABBIT AT END OF PERIOD
	Initial	40th day	Gain	Cotton seed meal	Iron salt		
852	1720	1890	170	600	13.65	40	Alive and normal.
853	1580	1780	200	600	13.65	40	Alive and normal.
Average.	1650	1835	185	600	13.65	40	

<sup>4</sup> Each of these rabbits was alive and normal after forty-one days' feeding, each having consumed 615 grams of cotton seed meal and 13.5 grams of iron salt.

Comparing the results obtained with this feed and those obtained with the whole meal feed without iron it will be noted: that the iron-fed rabbits consumed nearly five times the amount of meal which proved fatal to the average rabbit without iron, and more than twice as much as the greatest amount of meal necessary to kill the rabbit which ate the most meal; that the iron-fed rabbits have survived more than three times as long as the average rabbit without iron, and nearly twice as long as the hardest rabbit; that every rabbit fed iron and meal gained in weight and each rabbit fed meal without iron lost in weight.

It will be noted that each one of the twenty-two rabbits receiving cotton seed meal without iron died, that each of the eleven rabbits receiving iron along with cotton seed meal lived and gained in weight.<sup>3</sup>

These experiments lead us to suggest iron salts as an antidote to the toxicity of cotton seed meal.

Experiments are in progress with swine and results will be published later.

<sup>3</sup> Each of these rabbits was alive and normal after fifty-eight days' feeding, each having consumed 870 grams of cotton seed meal and 19.95 grams of iron salt.

## THE NECESSITY OF CERTAIN LIPINS IN THE DIET DURING GROWTH.

By E. V. MCCOLLUM AND MARGUERITE DAVIS.

(From the Laboratory of Agricultural Chemistry of the University of Wisconsin.)

(Received for publication, June 1, 1913.)

Whether or not the growing mammal must obtain certain lipins preformed in the diet, has up to the present time not been definitely determined. That bodies of this class can be dispensed with for a considerable period without interfering with normal growth, is now well established. Osborne and Mendel<sup>1</sup> have recently published data showing that young rats can grow normally during sixty days on rations containing but insignificant traces of ether-soluble matter.

That lipins of several kinds can be synthesized in large quantities in the animal body, is apparent from the experiments of McCollum, Halpin and Drescher,<sup>2</sup> who secured normal egg production in hens on a ration containing but very small amounts of ether-soluble substances. This observation has been fully confirmed by Fingerling<sup>3</sup> with ducks.

During the past year we have been engaged in a study of the influence of the composition and quantity of the inorganic content of the ration on growth in the rat.<sup>4</sup> In this work we have employed rations compounded of pure casein, carbohydrates, and salt mixtures made up of pure reagents, and the same rations in which a part of the carbohydrates was replaced by lard, with a considerable degree of success. Young rats have been found to be very sensitive to variations in the character of the salt mix-

<sup>1</sup> This *Journal*, xii, p. 81, 1912.

<sup>2</sup> McCollum, Halpin and Drescher: Proceedings of the American Society of Biological Chemists, this *Journal*, xi, p. xiii, 1912; also *ibid.*, xiii, p. 219, 1912.

<sup>3</sup> Fingerling: *Biochem. Zeitschr.*, xxxviii, p. 448, 1912.

<sup>4</sup> See McCollum and Davis: Proceedings of the American Society of Biological Chemists, this *Journal*, xiv, p. xl, 1913.

tures supplied, but with certain mixtures we have been able to obtain practically normal growth for periods varying from 70 to 120 days. Beyond that time little or no increase in body weight can be induced with such rations. These rats may remain in an apparently good nutritive condition on these rations for many weeks after growth ceases. That they are still capable of growth has been repeatedly demonstrated by changing to naturally occurring food-stuffs. That our animals, during their period of growth or during the period of suspension of growth which always accompanies long continued feeding of purified food substances, are in a physiological state which is nearly normal is evident from the fact that we have had three female rats produce young after being fed only casein, carbohydrates, lard and salt mixtures, for periods of 108, 127, and 142 days, respectively. These rats had made approximately normal growth for about eighty days on this ration. In none of these cases did the mothers produce enough milk to properly nourish the young, so that they were found to be decidedly undersized when seven to eighteen days old.

The fact that a rat of 40 to 50 grams in weight can grow normally during three months or more on such rations, then cease to grow but maintain its weight and a well nourished appearance for weeks and then resume growth on a ration containing certain naturally occurring food-stuffs would lead one to the belief that on these mixtures of purified food substances the animals run out of some organic complex which is indispensable for further growth but without which maintenance in a fairly good nutritive state is possible.

After numerous attempts to prevent the occurrence of growth suspension by nice adjustments between the various ingredients of our diets, we have found that the failure of rats to make further growth, after being brought to this "critical" point on mixtures of isolated food substances, is due to a lack of certain ether-soluble substances in the diet. These can be supplied by the ether extract of egg or of butter. The curves of a number of rats are presented to illustrate how a resumption of growth at about the normal rate results from the introduction of such ether-soluble substances into the diet after growth has ceased. It should be borne in mind that these experiments were intended primarily to furnish data as to the values of the salt mixtures



supplied by the rations and do not in all cases represent the highest degree of success attained by feeding these rations. The curves are selected entirely with a view to illustrating our almost invariable success in inducing a resumption of growth after complete suspension for a time on a ration which during eight weeks or more had sufficed for growth somewhat closely approximating the normal rate. The ether extracts employed were freed from ether *in vacuo* at temperatures below 60°C.

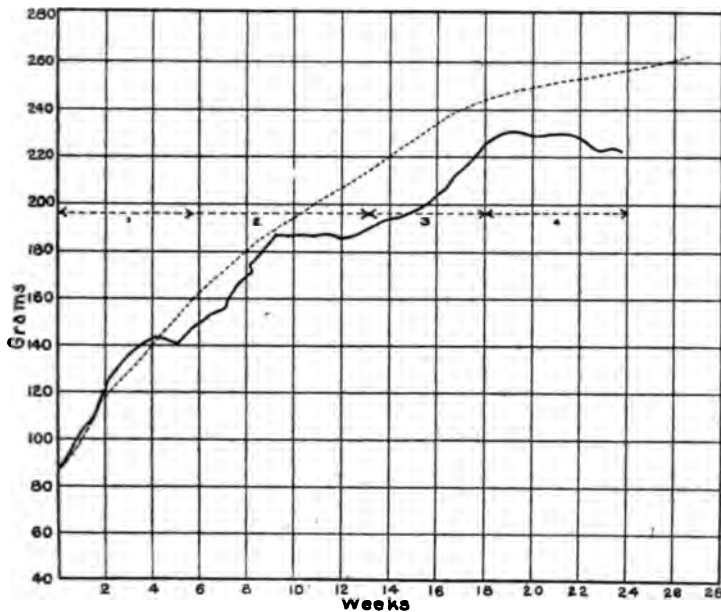


CHART I. *Rat 104* (male) shows the record of a rat which grew in a nearly normal manner during sixty-five days on a ration of purified food substances. The broken line represents the normal curve of growth. A suspension of growth occurred at this time and after four weeks without increase in body weight, 1 gram of ether extract of egg was added to the ration every other day, with the result that the animal gained 35 grams during the following forty-two days. The rations employed during the four periods of the experiment were as follows:

PERIOD I.	PERIOD II.	PERIOD III.	PERIOD IV.
per cent	per cent	per cent	
Salt mixt..... 6	Salt mixt..... 6	Salt mixt..... 6	Same as III but
Casein..... 18	Casein..... 18	Casein..... 18	without the
Lard..... 20	Lactose..... 15	Dextrine..... 74	ether extract
Lactose..... 20	Dextrine..... 59	Agar-agar... 2	of egg.
Starch..... 31	Agar-agar... 2	Ether extract of	
Agar-agar.... 5		egg, 1 gm. every	
		other day.	

The salt mixture employed in these rations consisted of:

	grams		grams
Calcium lactate.....	3.785	Dipotassium phosphate.....	3.648
Sodium citrate (anhydrous)...	3.296	Sodium chloride.....	3.430
Magnesium citrate(10.2% Mg)	1.298	Ferrie citrate.....	1.000
Potassium citrate.....	3.118		

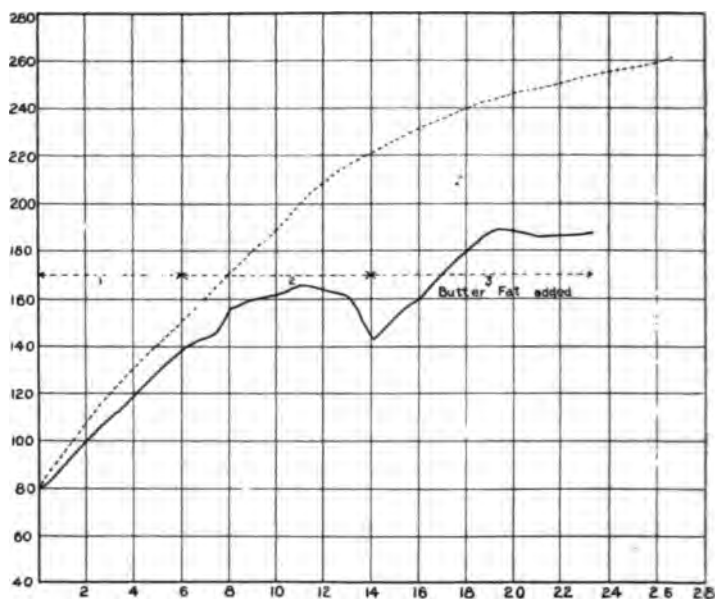


CHART II. Rat 141 (male) shows the record of a rat which grew continuously although slightly under normal rate during eighty days on a ration of purified food substances. There was at this time a complete suspension of growth and a rapid decline in body weight. The addition of 10 per cent of ether-soluble butter fat to the diet led to a prompt resumption of growth during the following thirty-five days, when the rat gained 50 grams.

The rations employed were as follows:

PERIOD I.	per cent	PERIOD II.	per cent	PERIOD III.
Salt mixture.....	6	Salt mixture.....	5	Same as II with butter
Casein.....	12	Casein.....	12	fat replacing part of
Lard.....	20	Lactose.....	20	dextrine.
Lactose.....	15	Dextrine.....	61	
Starch.....	42	Agar-agar.....	2	
Agar-agar.....	5			

The salt mixture employed consisted of:

	grams		grams
Sodium chloride.....	0.61	Calcium lactate.....	11.38
Dipotassium phosphate.....	17.00	Magnesium citrate(10.2%Mg)	23.42
Monocalcium phosphate.....	1.63	Ferric citrate.....	1.00

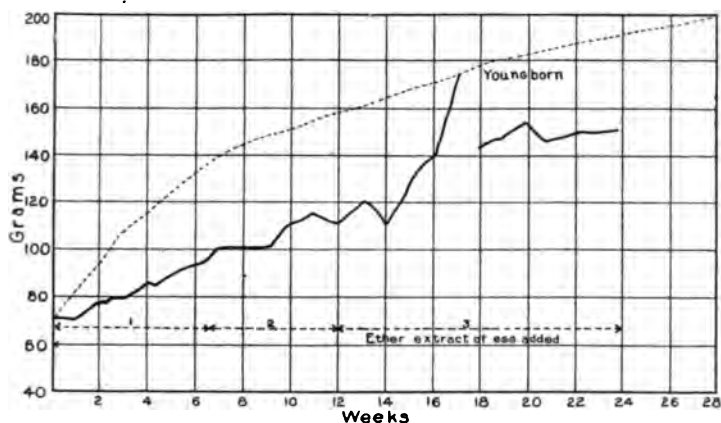


CHART III. *Rat 138* (female). This rat received a salt mixture which has induced much better growth in other rats. Her growth was but little over half normal during the first eighty-five days on a ration of purified food substances. She was with a male during this time but did not become pregnant. From the 86th day she was given 1 gram of ether extract of egg every other day. Growth was at once resumed and she became pregnant on the 14th day after the addition was made. On the 119th day of "artificial" feeding she gave birth to eight young which she suckled normally during twenty days. The aggregate weight of the young at the age of twenty days was 162 grams, the weight of the mother at this time being 155 grams. She is still in an excellent nutritive condition after one hundred and sixty-eight days and is continuing on the diet.

The rations employed were as follows:

PERIOD I.	per cent	PERIOD II.	per cent	PERIOD III.
Salt mixture.....	6	Salt mixture.....	8	Same as II with ether
Casein.....	18	Casein.....	18	extract of egg.
Lard.....	20	Lactose.....	20	
Lactose.....	15	Dextrine.....	52	
Starch.....	36	Agar.....	2	
Agar-agar.....	5			

The salt mixture employed was composed of:

	grams		grams
Calcium lactate.....	3.78	Potassium citrate.....	3.12
Sodium citrate.....	3.29	Magnesium citrate(10.2% Mg)	1.30
Sodium chloride.....	3.43	Ferric citrate.....	1.00
Dipotassium phosphate.....	3.65		

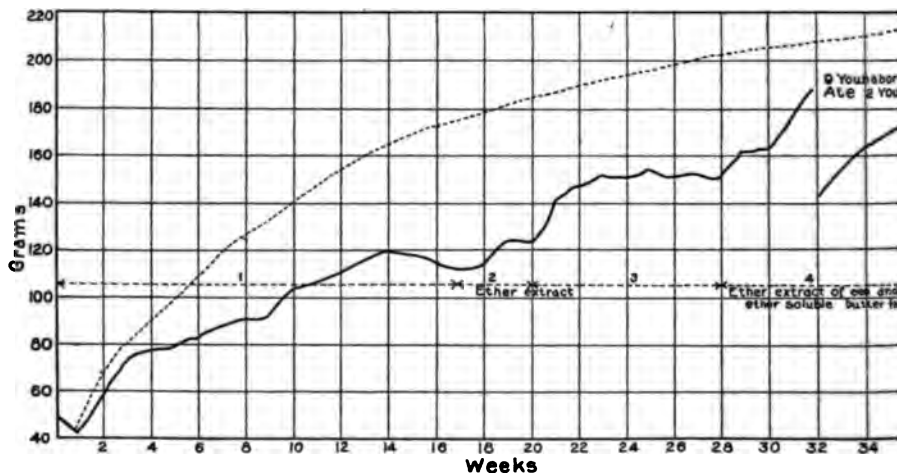


CHART IV. *Rat 104a* (female) shows the record of a rat during 257 days of nearly continuous growth. In period I the ration contained lard up to the 15th week. There was a suspension of growth near the end of this period. During period II the ration contained 5 per cent of ether extract of egg-yolk. No extract of egg was fed in period III which resulted in a second cessation of growth. In period IV growth was resumed and reproduction was attained. She ate two of the nine young produced and the others were removed. She is still growing on this ration and has attained about 80 per cent of the normal adult weight.

The rations employed in feeding this rat were as follows:

PERIOD I.		PERIOD II.		PERIOD III.		PERIOD IV.	
	per cent		per cent		per cent		per cent
Casein.....	18	Casein.....	18	Casein.....	25	Casein.....	18
Lard.....	25	Lactose.....	10	Lactose.....	20	Lactose.....	20
Lactose.....	10	Starch.....	62	Dextrine.....	40	Dextrine.....	40
Starch.....	37	Agar.....	5	Agar.....	5	Agar.....	2
Agar.....	5	Salt mixt.....	5	Salt mixt.....	10	Ether extract	
Salt mixt.....	5	Ether extract				of egg and	
		of egg add-				of butter....	10
		ed.				Salt mixt.....	8

There have been many slight modifications in the character of the inorganic content of the rations of this rat.

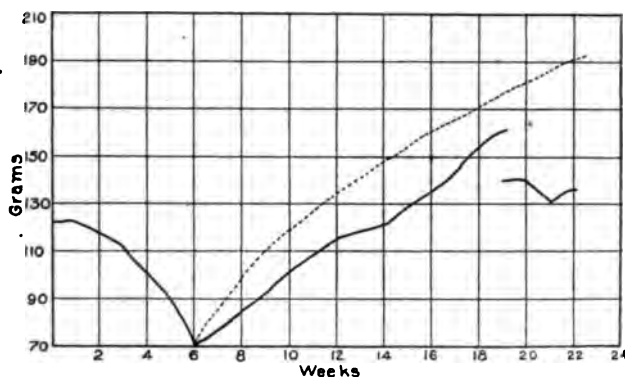


CHART V. *Rat 50* (female) is introduced to show what degree of success can be attained on diets containing no fats and but insignificant amounts of ether-soluble substances. The experiment covered one hundred and fifty-four days. During the first forty-two days the ration contained lard. There was a steady and rapid decline due apparently to the unsatisfactory character of the salt mixture fed, for on changing this there was a rapid recovery and nearly normal growth with the production of young after ninety-one days on a fat-free ration. The experiment was terminated because the mother ate two of her young and the third was only about half normal size on the 18th day (weight 10 grams). This one grew normally when placed with another mother suckling young of the same size.

The ration consisted of:

PERIOD I.		PERIOD II.		
	per cent		per cent	grams
Casein.....	18	Casein.....	18	NaCl..... 1.022
Lard.....	25	Lactose.....	20	MgSO <sub>4</sub> (anhydrous) 3.865
Starch.....	33.5	Dextrine.....	51	Mg citrate..... 7.000
Lactose.....	10	Agar-agar.....	5	K <sub>2</sub> HPO <sub>4</sub> ..... 0.168
Agar-agar.....	5	Salt mixt. used in		KH <sub>2</sub> PO <sub>4</sub> ..... 12.795
Salt mixt.....	7.5	period I, Chart		
		II.....	6	

The salt mixture employed in period I consisted of:

	grams		grams
Na <sub>2</sub> HPO <sub>4</sub> · 12H <sub>2</sub> O.....	5.052	CaH <sub>4</sub> (PO <sub>4</sub> ) <sub>2</sub> · H <sub>2</sub> O.....	1.880
Na citrate (anhydrous).....	2.866	CaSO <sub>4</sub> (anhydrous).....	0.168
Calcium lactate.....	25.377	Ferric citrate.....	1.000

We have seen this prompt resumption of growth after a period of suspension result from the introduction of ether extract of butter or of egg in about thirty animals and are convinced that these extracts contain some organic complex without which the animals cannot make further increase in body weight, but may maintain themselves in a fairly good nutritive state for a prolonged period. In no instance have we obtained such a result by the feeding of lard, or of olive oil. It is therefore not merely the absence of fats from the diet which causes the suspension of growth.

Whether the resumption of growth is the result of supplying in the ether extract of egg or of butter, some indispensable organic complex of the chemical nature of the lipins, or is the result of a stimulating action of some substance accompanying the lipins, cannot be decided from the data at present available. In a considerable number of instances we have fed lecithin or cholesterol with very doubtful results. Hopkins<sup>5</sup> observed that small quantities of milk added to rations of purified food substances, exert an influence on the growth of rats, which is out of all proportion to the added nitrogen or calorific value. Funk<sup>6</sup> and Suzuki, Shimamura and Odaki have isolated substances from rice polishings which exert a remarkable curative effect on birds suffering from polyneuritis as a result of exclusive feeding on polished rice.

<sup>5</sup> Hopkins: *Journ. of Physiol.*, xlv, p. 425, 1912.

<sup>6</sup> Funk: *Journ. of Physiol.*, xlv, p. 50, 1912; Suzuki, Shimamura and Odaki: *Biochem. Zeitschr.*, xliii, p. 89, 1912.

Funk has also obtained preparations from brain, yeast, and milk which have the same power.

The extensive literature on the remarkable physiological properties of certain fresh food-stuffs as contrasted with the cooked or preserved materials, in preventing or curing scurvy and beriberi, diseases arising from unsatisfactory diets, has been recently summarized by Cooper.<sup>7</sup> From the experimental data available it seems apparent that very young animals cannot be made to complete their growth on rations supplying only purified proteins, carbohydrates, fats, and salts. Our observation that ether extracts from certain sources improve the condition of animals on such rations, strongly supports the belief that there are certain accessory articles in certain food-stuffs which are essential for normal growth for extended periods.

It is interesting in this connection to correlate our observation on the physiological properties of ether extracts of butter or eggs, with those of Osborne and Mendel on the power of an animal to maintain itself on a ration containing gliadin as the only protein. While no growth is possible on this ration, notable increase in weight due to the building of young, can take place and a milk supply capable of normally nourishing the young can be produced. Through the agency of the ovary in egg production, or the mammary glands in milk production, the necessary accessory bodies essential to the proper nourishment of the young are readily synthesized by the animal cell. The young themselves have not the power to produce these syntheses for their own preservation when these unknown substances are lacking in the diet.

The further study of the nature of the "active" bodies in these extracts must of necessity require a great deal of time and labor, since preparations from butter or eggs made with solvents poorer than ether, and of ether extracts from other sources, the examination of their stability, etc., can be tested only on animals which have been grown as far as possible on rations of purified food substances, and have reached the stage of suspension of growth.

This work will be carried on as rapidly as circumstances will permit.

<sup>7</sup> Cooper: *British Med. Journ.*, No. 2727, p 722, 1913.





## THE BIOCHEMICAL RELATION BETWEEN PYRUVIC ACID AND GLUCOSE.

By H. D. DAKIN AND N. W. JANNEY.

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(Received for publication, June 1, 1913.)

In a recent communication by Paul Mayer<sup>1</sup> an unsuccessful attempt was made to demonstrate glucose synthesis from pyruvic acid in phlorhizinized animals. These negative results were the more surprising on account of Paul Mayer's earlier investigations<sup>2</sup> upon the effect and fate of pyruvic acid in the animal body. In these experiments it was shown in the most convincing fashion that glucose may be excreted in the urine on administering pyruvic acid to rabbits, that this effect is accompanied by hyperglycaemia, and finally that glycogen synthesis could be shown to follow consumption of pyruvic acid by previously starved rabbits.

As a result of his negative experiments Paul Mayer is inclined to the belief that a definite proof of the formation of glucose from pyruvic acid cannot be furnished by the use of glycosuric animals. His own experiments, which were completed before the publication of Mayer's second paper, lead to an entirely different conclusion. Moreover, we have learned from conversation with Dr. Mayer that he has obtained results essentially similar to ours. In mutual arrangement Dr. Ringer's results appear simultaneously with our own (p. 145).

We find that the sodium salt of pyruvic acid given by mouth to diabetic animals under suitable conditions may give rise to just as large an excretion of "extra glucose" as does administration of lactic acid itself. In these experiments we have used animals treated with phlorhizin and also human diabetics. When a pyruvate is administered subcutaneously to glycosuric

*chem. Zeitschr.*, xlix, p. 486, 1913.

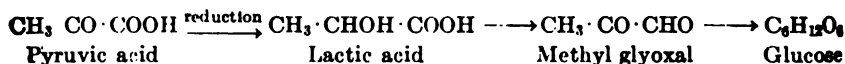
*ibid.*, xl, p. 441, 1912.

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dogs, especially to small animals, a definite but relatively small increase in "extra glucose" is noted. Incidentally we have observed that pyruvic acid which has undergone extensive polymerization on long standing may yield little or no glucose. It is therefore important for these experiments that the acid be freshly distilled before use.

A consideration of our results together with those of Paul Mayer leads us to the belief that glucose cannot be formed directly from pyruvic acid but that if the conditions are favorable for the reduction of the latter substance to lactic acid, then glucose may be produced. Such a hypothesis would harmonize with our observed experimental data and would be in entire accord with a hypothesis concerning glucose formation recently put forward by one of us in conjunction with Dudley.<sup>3</sup> It should be noted in this connection that Mayer has actually detected inactive lactic acid in the urine of normal rabbits receiving sodium pyruvate, and it is now known that both *d*- and *l*-lactic acids and methyl glyoxal may lead to the synthesis of glucose.

Our idea of the relationship between pyruvic acid and glucose may be gathered from the following scheme:



If the experimental conditions do not favor the initial reduction of pyruvic acid to lactic acid, it is probable that no synthesis of glucose can follow.

The experimental results are contained in the following tables. The general conditions were similar to those described in previous publications. The phlorhizin was given suspended in olive oil according to Coolen's method. In every case the excretion of acetoacetic acid and  $\beta$ -hydroxybutyric acid was followed, but no increase was observed to follow the administration of pyruvic acid. The figures are therefore omitted.

<sup>3</sup> This *Journal*, xiv, p. 555, 1913.

EXP.	PERIOD	GLUCOSE	NITROGEN	G:N	EXTRA GLUCOSE	SUBSTANCE GIVEN
I	I			3.28		
	II	11.42	3.48	3.31		
	III	19.53	3.38	5.78	8.75	12.5 gms. pyruvic acid.
	IV, V	9.81	3.15	3.11		
	VI	16.56	3.21	5.16	8.46	10.7 gms. lactic acid.
	VII	12.15	3.08	3.94		
II	I	25.38	7.83	3.24		
	II	27.03	7.83	3.45		
	III	25.07	6.43	3.88	3.40	12 gms. pyruvic acid.
	IV	25.91	7.60	3.41		
	V	29.92	7.29	4.14	5.35	12 gms. pyruvic acid.
III	I	28.19	8.93	3.16		
	II	32.15	8.12	3.96	6.65	10 gms. pyruvic acid.
	III	27.52	8.82	3.12		
	IV	26.29	8.91	2.95	0	15 gms. polymerized pyruvic acid.

EXP.	PERIOD	GLUCOSE	NITROGEN	CARBOHY- DRATE IN DIET AS GLUCOSE	SUBSTANCE GIVEN
IV	I	4.39	11.56	13.2	
	II	8.28	12.25	16.8	
	III	5.73	9.90	16.1	
	IV	7.12	10.43	17.7	
	V	21.30	11.72	17.0	44 gms. pyruvic acid in 5 portions.
	VI	21.20	15.34	19.7	
	VII	5.72	10.97	17.9	
	VIII	5.92	10.22	17.7	
	IX	20.37	9.37	17.9	37 gms. lactic acid in 7 portions.
	X	18.55	8.11	16.3	
	XI	12.75	11.52	18.4	

**EXPERIMENT I.** Dog weighing 12 kgms. Sodium pyruvate and lactate given by stomach tube. Urine collected in 6-hour periods. Using  $G:N = 3.22$  it may be calculated that 12.5 grams of pyruvic acid gave 8.75 grams "extra glucose," while 10.7 grams lactic acid gave 8.46 grams.

**EXPERIMENT II.** Bitch weighing 8 kgms. Urine collected in 12-hour periods. Sodium pyruvate given subcutaneously in period II, by mouth, in period V. Using  $G:N = 3.37$ , the subcutaneous administration of 12

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grams of pyruvic acid appears to give 3.4 grams of "extra glucose," while the same amount given by mouth yielded 5.35 grams.

EXPERIMENT III. Dog weighing 19 kgms. 10 grams of pyruvic acid as sodium salt given by mouth gave 6.65 grams of "extra glucose." 15 grams of polymerized pyruvic acid given subcutaneously led to no additional glucose excretion.

EXPERIMENT IV. Female. Moderate case of diabetes mellitus. Multiple sclerosis and slight tubercular involvement of right pulmonary apex. During a preliminary period of nine days when the total carbohydrate consumption averaged 80 grams per day, the average daily glucose excretion was 32 grams. A large increase in glucose excretion is seen to follow the administration of both sodium pyruvate and lactate.

EXPERIMENT V. A mild case of diabetes mellitus, similar to the above. Only a trifling increase (2-7 grams) in glucose excretion followed the consumption of sodium pyruvate (28 grams) and sodium lactate (5.3 grams). The analyses show little of interest and are not reproduced.

## THE RELATION OF DIETS AND OF CASTRATION TO THE TRANSMISSIBLE TUMORS OF RATS AND MICE.

By J. E. SWEET, ELLEN P. CORSON-WHITE AND G. J. SAXON.

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(Received for publication, June 3, 1913.)

The study of the transmissible tumors of animals has brought to the attention of every worker in this field, facts of curious variations in the reaction to the tumor of animals obtained from different sources. A transmissible tumor of the mouse, for instance, can be brought to a quite constant degree of virulence, as measured both by the percentage of positive inoculations and by the rate of growth, on a given lot of mice; but mice obtained from a different source may show a marked diminution in the number of "takes" and an increase in the number of retrogressions. After a series of inoculations in the new strain of mice, especially if previous to inoculation the mice have been subjected to the same conditions as the animals in which the standard of virulence had been determined, the virulence can gradually be brought back to the original standard.<sup>1</sup> Haaland<sup>2</sup> found, for example, that a tumor which gave 100 per cent takes in Berlin mice, gave only 24 per cent in Hamburg mice, and was practically harmless in Christiana mice. An apparently related phenomenon is noted in the so-called spontaneous tumors of mice; a breeder may find a large number of tumors among his animals but they can only rarely be successfully transplanted.

A large number of the subcutaneous adenocarcinomata of mice are not transmissible even into mice of the same strain, while other tumors of similar histological structure are transplanted easily.<sup>3</sup> In all cases the transmissible tumor is most easily transplanted to the original tumor-bearing animal and next to animals

<sup>1</sup> Cuénot and Mercier: *Compt. rend. de l'Acad. des Sci.*, cxlvii, p. 1003, 1908.

<sup>2</sup> Haaland: *Berl. klin. Wochenschr.*, xlv, p. 713, 1907.

<sup>3</sup> Loeb: *International Clinics*, p. 121, 1907.

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of the same strain. Perhaps the most striking case of this peculiarity of tumors is recorded in the work of Rous,<sup>4</sup> with a transmissible tumor of the fowl. This tumor, found in a pure blood Plymouth Rock hen was at first only transferable to birds of precisely the same strain as that in which the original tumor occurred, indeed only to birds from the same brood as the original tumor fowl.

These facts have naturally engaged the attention of many workers, and numerous studies have been made of the factors which might be expected to influence this condition of susceptibility or immunity of the host. Loeb<sup>5</sup> finds that little if any influence seems to be exerted by the sex of the animal on the results with the transplantable tumors, while the spontaneous tumors of mice are found almost exclusively among the females.<sup>6</sup> Haaland,<sup>7</sup> Cuénot and Mercier,<sup>8</sup> Uhlenhuth and Weidanz<sup>9</sup> all state that the growth of a neoplasm is retarded during pregnancy and may even cease. This retardation has been thought to be due to the tax on the mother during gestation, and to be comparable to the unfavorable soil for tumor inoculation found in sick or ill-nourished animals.<sup>10</sup> This explanation is however probably not true, for under normal conditions pregnancy is a stimulus to growth and to nutrition.<sup>11</sup>

The age of the animal seems to exert a certain influence in so far that animals one-half to three-quarters grown seem to be the most favorable hosts for the transplantation of tumors.<sup>12</sup> The factor of heredity has been studied but the results are not very definite as yet.<sup>13</sup> We have been able to make a few observations

<sup>4</sup> Rous: *Journ. of Exp. Med.*, xiv, p. 696, 1910.

<sup>5</sup> Loeb: *loc. cit.*

<sup>6</sup> Jobling: *Monograph of Rockefeller Institute*, i, p. 174, 1910.

<sup>7</sup> *Loc. cit.*

<sup>8</sup> Cuénot and Mercier: *Compt. rend. soc. biol.*, lxxvii, p. 736, 1909.

<sup>9</sup> Uhlenhuth and Weidanz: *Arbeit. a. d. Kais. Gesundheitsamte*, xxx, p. 434, 1909.

<sup>10</sup> Joannovics: *Wien. klin. Wochenschr.*, 1912, p. 36.

<sup>11</sup> Rous: *Journ. of Exp. Med.*, xiii, p. 248, 1911.

<sup>12</sup> Bradford, Murray, Haaland and Bowen: *Third Scientific Report*, Imperial Cancer Research Fund, 1900, p. 265.

<sup>13</sup> Tyzzer: *Journ. of Medical Research*, xxi, p. 519, 1909; Cuénot and Mercier: *Compt. rend. soc. biol.*, lxix, p. 645, 1910; Loeb and Fleischer: *Zentralbl. f. Bakt.*, lxxvii, p. 3, 1912.

upon the relation of heredity to the rat tumor, through the generous coöperation of the Wistar Institute. Our rats have been discarded litters from certain breeding experiments conducted by the Wistar Institute, and these litters have been kept as units in our work. The susceptibility or immunity has been, almost invariably, not a matter of individual animals but of the entire litter; the degree of susceptibility has also proven to be a question of the litter, not of the individual. For example, the same tumor may not take at all in one litter, prove slightly virulent for a second litter as measured by the rate of growth, and extremely virulent for a third litter. The suggestion to make use of the litter unit was offered to us by Dr. H. H. Donaldson, and, while our observations on this line are not complete, we are inclined to agree with those of larger experience in breeding experiments, that observations of litter units may in tumor work also, prove of greater value than observations of large numbers of mixed animals.

A few investigators have experimented to some extent upon the relation of diet to the growth of transplantable tumors. Up to the time of the beginning of our work, however, the literature contained but few such references and the experiments described seem to have been of a somewhat desultory character. Jensen<sup>14</sup> suggested that diet might possibly influence the recurrence and metastases. Haaland<sup>15</sup> discusses the effect of light, heat, moisture, etc., and concludes that diet is probably of the most importance. He found that mice on a diet of hemp seed, bread and milk, with some oats, were more susceptible to a certain sarcoma than those on ordinary diet; while those animals limited to a diet of bread and oats were the least susceptible. Stahr<sup>16</sup> studied the effect of diet on a home strain of mice and a resistant strain from another locality, finding that a diet of hemp seed and milk rendered the animals less susceptible than the eating of bread and water. Moreschi<sup>17</sup> made careful studies on diet with reference to the changes in weight of mice and the rate of growth of

<sup>14</sup> Jensen: *Zeitschr. f. Krebsforschung*, xx, p. 682, 1909.

<sup>15</sup> Haaland: *loc. cit.*

<sup>16</sup> Stahr: *Centralbl. f. allg. Path. u. path. Anat.*, xx, p. 628, 1909.

<sup>17</sup> Moreschi: *Zeitschr. f. Immunitätsforschung*, etc., ii, p. 651, 1909.

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their tumors, as did Medigreceanu.<sup>18</sup> The unfavorable influence of poor nutrition as brought about by intercurrent disease upon the rate of growth of the transplanted tumor is a matter of general observation. Moreschi<sup>19</sup> found that in animals with the food supply reduced to a minimum compatible with life, with no evidence of previous or obvious illness, tumors develop less frequently and grow more slowly than in the controls. Our own work along the line of the influence of diet was based upon the work of Mendel and Osborne,<sup>20</sup> who found in their studies of the effects of feeding rats with combinations of pure vegetable proteins a number of diets which completely retarded the normal growth of the animal, although the general condition seemed entirely normal. In other words their animals were not starved in any sense except a very specific one—certain elements necessary to normal growth were lacking. It is not the place in this paper to discuss their findings of such importance to our knowledge of nutrition and growth. We gladly accepted their work as an opportunity to study this relation of nutrition and growth to a transplanted tumor. To put the question more concisely, regardless of whatever may be the ultimate cause of the cancer growth, could a cancer grow in a body rendered incapable of normal cell growth?

In our experiments we systematically used one-half or three-quarter grown rats and mice; two weeks were allowed to pass between the beginning of the special diet or the operative interference and the inoculation of the animal. The control diet was made up of bread, oats, wheat, rice, corn, sunflower seed and water. The special diet was made up on a basis of the diet used by Mendel and Osborne for rat 179.<sup>21</sup> Preliminary experiments showed that a gluten mass obtained by careful washing of wheat flour could be satisfactorily used in place of the chemically pure substances studied by Mendel and Osborne. By this we mean that the animals could be kept at an almost constant weight—some indeed absolutely constant—for the periods of time neces-

<sup>18</sup> Medigreceanu: *Berl. klin. Wochenschr.*, xlvii, p. 772, 1910; *Proc. Royal Society*, lxxxii, p. 286, 1910.

<sup>19</sup> *Loc. cit.*

<sup>20</sup> Mendel, Osborne and Ferry: *Zeitschr. f. physiol. Chem.*, lxxx, p. 307, 1912.

<sup>21</sup> *Zeitschr. f. physiol. Chem.*, lxxx, p. 318, 1912.



sary for tumor work. We have no doubt that our results would have been more marked had we made use of chemically pure glutenin and gliadin, but since the gluten in our hands effectively prevented growth and because of the difficulty of obtaining a sufficient quantity of chemically pure substances for feeding large series of animals we felt obliged to content ourselves. The diet was therefore made up of gluten 18 per cent, starch 24.5 per cent, lard 27 per cent, lactose 23 per cent, salt mixture 2.5 per cent, agar 5 per cent.

The original tumors used in our work were obtained from the Rockefeller Institute through the courtesy of Dr. Peyton Rous and were the Flexner-Jobling adenocarcinoma of the rat, and the Rockefeller Institute mouse tumor No. 33, a carcinoma. In every experiment bits of the same tumor were used for the inoculation of the controls and the animals under special treatment.

TABLE I.

DIET	NUMBER OF MICE	NUMBER OF TAKES	LATENT PERIOD IN DAYS	AVERAGE DIAMETER IN MILLIMETERS OF TUMORS AFTER 16 DAYS
Normal.....	25	23	7	2.2
Mendel-Osborne.....	25	9	9	0.9

At the end of sixteen days this experiment was divided as follows: the nine positive animals on the Mendel-Osborne diet were continued on this diet; four days later, or twenty days after inoculation, five of these nine tumors had apparently completely retrogressed. Ten days later, or thirty days after inoculation, two of the remaining four had retrogressed. One of the two mice left died on the fifty-second day with a tumor measuring 5.2 mm. in its largest diameter. The remaining mouse on the fifty-second day had a tumor 4 mm. in diameter. It was then changed to a normal diet, and at the end of thirty days was possessed of the tumor shown in the photographs on the following page.

The sixteen negative mice of this series were changed at the end of sixteen days to a normal diet; three developed tumors five days later or twenty-one days after inoculation. Ten days

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later or twenty-six days after inoculation these tumors had a diameter of 1.7 mm.; twenty days later or thirty-six days after inoculation 7 mm., and thirty days later or forty-six days after inoculation, 21 mm. The latent period of twenty-one days in these animals is noteworthy. The 23 mice of the normally fed group were divided as follows:

TABLE II.

DIET	NUMBER OF MICE	SIZE OF TUMOR IN MILLIMETERS AFTER INOCULATION			RETROGRA- SSIONS
		16 days	20 days	30 days	
Normal.....	11	2.2	4.8	19.7	3
Mendel-Osborne.....	12	2.2	3.4	5.1	5

This experiment was repeated by inoculating one hundred mice, fifty males and fifty females, with bits of the same tumor and dividing them as follows:

TABLE III.

DIET	NUMBER OF MICE	TAKES	LATENT PERIOD IN DAYS	AVERAGE DIAMETER OF TUMOR IN MILLIMETERS, AFTER		
				10 days	20 days	30 days
Normal.....	25 males	19	7	6.7	15.9	19.3
Normal.....	25 females	15	7	4.5	9.2	15.7
Mendel-Osborne.....	25 males	7	12		2.4	5.3
Mendel-Osborne.....	25 females	8	10	1.0	3.1	8.4

The results with the rat tumor have been of the same general trend; as others have observed, however, work with the rat tumor is less satisfactory than with the mouse tumor, the rat tumor showing many unexplainable irregularities in its behavior. Because of this irregularity of behavior we have included in the following table only those rats on the Mendel-Osborne diet in which there was a definite "take."

TABLE IV.

DIET	NUMBER OF RATS	TAKES	LATENT PERIOD	AVERAGE DIAMETER IN MILLIMETERS AFTER			RETROGRESSIONS
				10 days	20 days	30 days	
Normal.....	59	31	9 days	6.1	9.8	14.7	0
Mendel-Osborne....	59	31	9 days	4.9	7.2	10.0	8

It would seem fair to conclude from these experiments that the number of successful transplantations can be markedly reduced by a diet which prevents normal body growth. The rate of growth of the tumor is much slower and the number of retrogressions is high on such a diet. The effect of diet would seem to be more marked upon the receptivity of the host, as shown in the diminished number of takes and the increase of latent period seen in Table III, and yet that this diet can influence a tumor which has already started is seen in Table IV where nearly 25 per cent of retrogressions occurred on a special diet, with no retrogressions in the controls.

We have further experimented with the diets described by Reid Hunt<sup>22</sup> as influencing the thyroid gland. An exclusive diet of

<sup>22</sup> Reid Hunt: Hygienic Laboratory Bulletin 69, 1910.

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oats and water or 2 per cent KI solution is supposed to accelerate the thyroid function, a fact in line with the findings of Watson,<sup>22</sup> who found the thyroid enlarged in young rats fed wholly on oats. The second diet, eggs and milk, is said to retard or inhibit thyroid function. This diet however proved to be so detrimental to the health of the animals, both rats and mice, that only a very few were able to thrive; these few, after a preliminary diarrhoea, grew fat and seemed to be in the best of health.

TABLE V.

DIET	NUMBER OF EXPERI- MENTS	NUM- BER OF MICE	TAKES	LATENT PERIOD IN DAYS	AVERAGE DIAMETER IN MILLIMETERS OF TUMORS AFTER		
					10 days	20 days	30 days
Normal.....	14	250	103	7	2	7	10
Thyroid accelerating...	14	250	166	7	4	12	27
Thyroid inhibiting.....	5	75	12	12		4	7

TABLE VI.

DIET	NUMBER OF EXPERI- MENTS	NUMBER OF RATS	TAKES	LATENT PERIOD IN DAYS	AVERAGE DIAMETER IN MILLIMETERS OF TUMORS AFTER	
					20 days	30 days
Normal.....	7	74	52	14	6.1	27.0
Thyroid accelerating...	3	50	36	10	7.4	31.4
Thyroid inhibiting.....	3	26	10	15	4.0	15.0

Whether or not the interpretation of the experiments from which it was concluded that the thyroid gland is influenced by these diets be correct, we may at least conclude that here again as with the Mendel-Osborne diets the transplantable tumor can be influenced by the diet. It is in accord with our idea that the influence of diet may be exerted through the ductless glands, and it was from this point of view that we made the experiments described above.

Our next studies were undertaken with the specific purpose of studying the effect of the removal of certain of the ductless glands

<sup>22</sup> Watson: *Lancet*, i, p. 985, 1907.

upon the transplanted tumor. Our work has been limited to castration of the male animals chiefly because of our knowledge of the work of Dr. Hatai (now in press from the Wistar Institute) upon the relation of castration to the other ductless glands. Hatai finds after castration a persistence of the thymus and an increase of 74 per cent in the weight of the hypophysis. Studies of the effect of castration upon tumors have been made before. Graf<sup>24</sup> made five experiments using sixty-six castrate males, forty normal males, fifty-six castrate females, and thirty-six normal females, and inoculating with the most malignant tumor in his possession. He concludes that there is no essential difference in the tumor takes or in the energy of growth between the normal and the castrated animals. It seems to us that his experiment was hardly properly designed to bring out any differences, since changes in the nature of an increase of susceptibility might be masked by using a tumor which gives 100 per cent of positive inoculations in the controls. Rohdenburg, Bullock and Johnston<sup>25</sup> removed the thyroid, thymus, spleen and testes, and inoculated after varying intervals with a tumor. Their results after castration show only a marked shortening of the latent period.

TABLE VII.

	NUMBER OF EXPERI- MENTS	NUMBER OF ANIMALS	TAKES	LATENT PERIOD IN DAYS	AVERAGE DIAMETER IN MILLIMETERS OF TUMORS AFTER		
					10 days	20 days	30 days
Normal mice.....	3	73	30	7	1.8	5.0	12.0
Castrated mice.....	3	73	54	7	2.0	16.0	31.0
Normal rats.....	2	15	5	10	4.0	8.0	18.0
Castrated rats.....	2	14	9	10	7.0	14.0	47.0

It would seem from our experiments that the removal of the testes in some way renders an animal a more receptive host as seen by the increased takes and the more rapid growth.

In order to make a more satisfactory comparison of the effects of the diets and of castration we did the following two experiments, the results of which are embodied in Table VIII. Two

<sup>24</sup> Graf: *Centralbl. f. Path.*, xl, p. 17, 1909.

<sup>25</sup> Rohdenburg, Bullock and Johnston: *Arch. of Internal Med.*, vii, p. 491, 1911.

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series of 150 mice in each series, each series of 150 mice being inoculated with the same tumor, were divided as seen in the table.

TABLE VIII.

	NUM- BER OF MICE	TAKES	PER CENT OF TAKES	LATENT PERIOD IN DAYS	AVERAGE DIAMETER IN MILLIMETERS OF TUMORS AFTER		
					10 days	20 days	30 days
Normal diet.....	75	56	74.6	7	2.4	8.1	22.8
Mendel-Osborne diet ..	75	18	24.0	14		2.1	4.8
Thyroid accelerating ..	50	40	80.0	7	3.1	9.4	28.9
Thyroid inhibiting .....	16	2	12.5	17		3.8	5.3
Castrates.....	50	44	88.0	7	4.9	17.4	43.1

Certain facts of clinical experience formed the basis of the work outlined in this paper. We know for instance of the relation of the thyroid to growth, as seen in cretinism; of the relation of the pituitary to acromegaly and gigantism and of the control of the pituitary over the only instance of embryonic cell reproduction which persists in adult life, spermatogenesis and oögenesis. Finally it is a clinical fact that cancer notwithstanding many exceptions is a disease of the menopause, that period of life when certain of the ductless glands lose their normal function, this loss entailing related changes in the whole chain of the interrelated functions of the ductless glands. A further thought was that such a study might finally lead to some light on the question of the parasitic nature of the disease, for it would seem difficult to assume that a parasitic process would obey the laws of normal growth. Finally, as in tuberculosis, it may be found with cancer that the best treatment will be one not directed primarily against the disease process, but directed toward the stimulation of the normal protective functions of the body.

It may very fairly be objected that in no instance, except perhaps that of castration, in which our results do not entirely agree with those found by others, have we shown that any influence of our experiments is in fact brought about through the function of the ductless glands. We grant this objection and will simply leave our hypothesis stand for the present, since it at least shows that our work has been directed toward a definite goal. In what-

ever manner it is to be explained, we conclude from our experiments that:

1. The susceptibility of rats and mice to the transplantable tumors may be influenced both positively and negatively by proper diets.
2. The rate of growth of the transplanted tumors can be positively or negatively influenced by proper diets.
3. Castration of the male renders the animal more receptive to the transplanted tumor and the rate of growth of the tumor is increased.





## ON CEREBRONIC ACID.

### THIRD PAPER.

#### ITS BEARING ON THE CONSTITUTION OF LIGNOCERIC ACID.

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(Received for publication, June 9, 1913.)

In our study of cerebronic acid<sup>1</sup> an acid of the same composition and melting point as lignoceric acid was obtained by the action of alkaline potassium permanganate upon the potassium salt of cerebronic acid. It was pointed out at the time that this acid might be identical with lignoceric acid. Since then we have prepared derivatives of this new acid, which prove to be the same as those given in the literature for lignoceric acid.<sup>2</sup>

It has been shown that cerebronic acid has a normal carbon chain by reduction to pentacosan, melting at 53°–54°. This was further confirmed by the isolation of a small amount of an hydrocarbon melting at 51°–52° from the reaction product of hydroiodic acid upon the new acid,  $C_{25}H_{48}O_2$ . Thus it is quite definitely established that the new acid has a normal carbon chain. Since this acid is identical with lignoceric acid, then lignoceric must be considered a normal acid of twenty-four carbon atoms.

The following gives a comparison of the acids and their derivatives:

	Lignoceric acid	Acid from Cerebronic acid
	m. p.	m. p.
Acid.....	80.5°–81°	80°–81°
Methyl ester.....	55°	55°
Ethyl ester.....	57°–58°	58°
Lead salt.....	117°	116°–117°

<sup>1</sup> Levene and Jacobs: this *Journal*, xii, p. 381, 1912; Levene and West: *ibid*, xiv, p. 257, 1913.

<sup>2</sup> Hell and Hermann: *Ber. d. deutsch. chem. Gesellsch.*, xiii, p. 1713, 1880; Kreiling: *ibid*, xxi, p. 880, 1888.

## EXPERIMENTAL.

*Methyl ester.*

The methyl ester of the new acid was prepared by boiling 2 grams of the acid, 100 cc. of absolute methyl alcohol and 4 cc. of concentrated sulphuric acid four hours on the water bath. The ester which separated on standing over night at 0°, was recrystallized from methyl alcohol twice, then from acetone and finally dried in the chloroform bath for two hours. It melted at 58°. Hell and Hermann give 56.5°–57°; Kreiling, 58°.

0.1200 gram of substance gave 0.3450 gram CO<sub>2</sub> and 0.1416 gram H<sub>2</sub>O.

	Calculated for C <sub>24</sub> H <sub>40</sub> O <sub>7</sub> .CH <sub>3</sub> :	Found:
C.....	78.53	78.41
H.....	13.09	13.20

*Ethyl ester.*

The ethyl ester was prepared in the same way as the methyl ester. It was recrystallized from ethyl alcohol three times, when it melted at 55–56°. It was then evaporated to dryness with a slight excess of sodium methylate and extracted with ether to remove any trace of free acid and recrystallized from acetone, when it melted at 55°. Both Hell and Kreiling give 55° as the melting point of the ethyl ester.

0.1194 gram of substance gave 0.3386 gram CO<sub>2</sub> and 0.1408 gram H<sub>2</sub>O.

	Calculated for C <sub>26</sub> H <sub>48</sub> O <sub>7</sub> .C <sub>2</sub> H <sub>5</sub> :	Found:
C.....	78.79	79.12
H.....	13.13	13.20

*Lead salt.*

The lead salt was prepared by treating a hot methyl alcohol solution of the acid with lead acetate in the same solvent as long as a precipitate formed. A drop of ammonia was then added to complete the precipitation. This was filtered off when cool, washed with warm methyl alcohol and dried in vacuum. When heated quickly it softened at about 110° and melted at 116°–117°. Hell and Hermann give the melting point as 117°.

0.1200 gram substance gave 0.2694 gram CO<sub>2</sub> and 0.1073 gram H<sub>2</sub>O.

	Calculated for (C <sub>21</sub> H <sub>37</sub> O <sub>2</sub> ) <sub>2</sub> Pb:	Found:
C.....	61.19	61.23
H.....	10.06	10.01



## COLORIMETRIC DETERMINATION OF EPINEPHRINE IN DESICCATED SUPRARENAL GLANDS.

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(Received for publication, June 3, 1913.)

In a previous paper<sup>1</sup> from this laboratory it was shown that the color developed by heating desiccated suprarenal glands with a faintly acidified solution of potassium iodate could be used as an approximate measure of the epinephrine content of the sample, as determined by the blood pressure method. On the basis of the experiments then made, details were proposed for a rapid colorimetric estimation of the physiological activity of commercial desiccated suprarenal glands.

Although the iodate reagent was selected as the best of quite a number that were tried, it possessed the disadvantage of yielding slightly variable shades of color with different samples of glands. The method, therefore, could not be applied with equal success to all samples. In spite of this defect the approximate values that were obtained with nine samples of glands showed that even this more or less imperfect method could be made to serve a useful purpose, in differentiating between inferior samples and those of average physiological activity.

Since the publication of our paper, other and more delicate color reactions for epinephrine, have been proposed. Of these the phosphotungstic acid reagent of Folin and Denis<sup>2</sup> and the gold chloride reagent for which great sensibility has recently been claimed by Gautier<sup>3</sup> are particularly worthy of attention.

<sup>1</sup> Hale and Seidell: *Amer. Journ. of Pharm.*, lxxiii, pp. 551-8, (Dec.) 1911.

<sup>2</sup> Folin and Denis: this *Journal*, xii, p. 239, 1912; Folin, Cannon and Denis: *ibid.*, xiii, pp. 477-483, 1913.

<sup>3</sup> Gautier: *Compt. rend. de la soc. de biol.*, lxxiii, pp. 564-565, 1912.

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*Phosphotungstic acid reagent of Folin and Denis.* In their paper with Cannon the authors give minute details in regard to the estimation of the epinephrine in fresh suprarenal glands. It was, therefore, only necessary to introduce the modifications required for determinations in dried instead of the moist glands. The weights of moist glands which had been used varied from 0.164 to 11.69 grams, hence it was concluded that 0.5 gram of desiccated material would be a convenient amount to use. The determinations, the results of which are shown in table I, were made as follows: Fifteen cubic centimeters of  $\frac{N}{10}$  hydrochloric acid and 0.5 gram of the sample of desiccated suprarenal gland were mixed and after about an hour, 45 cc. of water were added and the solution heated to the boiling point; 5 cc. of 10 per cent sodium acetate solution were then added and the boiling continued a minute or so; after cooling, the solution was diluted to 100 cc. and well mixed. To 5 cc. of the clear supernatant liquid, 2 cc. of the phosphotungstic acid reagent and 20 cc. of saturated sodium carbonate solution were added. The mixture was diluted to 100 cc., thoroughly mixed and the intensity of its blue color estimated by comparison in a Duboscq colorimeter with standards made simultaneously from 0.1 per cent uric acid solution.<sup>4</sup>

The phosphotungstic acid method as developed by its authors certainly has much to commend it. The fine blue color yielded by the uric acid standard and the gland samples appears to be of exactly the same shade and the relative intensities of different solutions can be accurately estimated. The sensitiveness of the reaction is such that the developed color is not sensibly modified by any extractive material derived from the sample itself, as appears to be the case with the iodate reaction. Therefore, disturbing differences of shades of color are not observed with different commercial samples. The exceptional sensitiveness also permits an apparent accuracy to the second and possibly even

<sup>4</sup> In the epinephrine paper of Folin, Cannon and Denis (*loc. cit.*) the authors omit to state that alkali must be added to effect solution of the uric acid. In a previous paper by Folin and Macallum, Jr. (*this Journal*, xiii, p. 366, 1912), it is directed that 0.25 gram uric acid be dissolved with the aid of 25 cc. of 0.4 per cent  $\text{Li}_2\text{CO}_3$  and the solution diluted to 250 cc. Following these directions I found that all of the uric acid did not dissolve in two and one-half hours and, therefore, added a second 25 cc. of  $\text{Li}_2\text{CO}_3$  solution in order to obtain a clear solution.

TABLE I.

*Percentages of epinephrine in commercial desiccated suprarenal glands as determined by the phosphotungstic acid method of Folin and Denis.*

SAMPLE NO.	SOURCE	AVERAGE OF AT LEAST 5 READINGS IN DUBOSCQ COLORIMETER		CALC. GMS. EPINEPHRINE IN ALIQUOT USED	CALC. PER CENT EPINEPHRINE IN SAMPLE
		Sample	Standard		
362	Sheep	25	14.1	0.000188	0.752
363	Sheep	25	14.0	0.000187	0.748
365	Sheep	25	11.0	0.000147	0.588
366	Sheep	25	4.2	0.000056	0.224
367	Sheep	25	12.9	0.000172	0.688
368	Sheep	25	6.2	0.000083	0.332
369	Sheep	25	8.2	0.000109	0.436
370	Sheep	25	1.9	0.000025	0.100
416	Sheep	25	6.5	0.000087	0.348
417	Beef	25	17.2	0.000229	0.916
418	Hog	25	5.7	0.000076	0.304
572	Beef	25	14.0	0.000187	0.748
573	Beef	25	12.0	0.000160	0.640
574	Beef	25	17.1	0.000228	0.912
575	Hog	25	5.2	0.000069	0.276
576	Hog	25	2.5	0.000031	0.124
577	Hog	25	5.6	0.000075	0.300
578	Sheep	25	6.9	0.000092	0.368
579	Sheep	25	4.3	0.000057	0.228
580	Sheep	25	6.2	0.000083	0.332

to the third decimal place. The method will no doubt be found of particular value in the examination of samples of known origin, especially those of which only very small amounts are available and in cases where the percentage of contained epinephrine is exceptionally minute.

Of the samples upon which results are reported in table I, the first eight are those for which data are given in our previous paper.<sup>5</sup> It will be noted that the phosphotungstic acid reagent has in general given slightly higher results than the iodate reagent, but still not as high values as obtained by the physiological assay. There is one important exception, however, in the case of sample No. 370 which the phosphotungstic acid reagent shows to be two

<sup>5</sup> *Loc. cit.*

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and one-half times as active as indicated by its action on the blood pressure. The comparative results reported by Folin, Cannon and Denis upon their samples show, on the other hand, a remarkable agreement between the phosphotungstic acid reagent and the physiological method of assay. It should be noted, however, that these authors used in all cases the same gland extract for both determinations, whereas in our experiments separate weighed samples of the glands were extracted in the particular manner adopted for each method.

From the standpoint of the analyst desiring a control method for commercial desiccated suprarenal glands, the phosphotungstic acid reagent seems to possess several disadvantages. The fact that uric acid and quite a number of other compounds yield exactly the same shade of blue color as epinephrine, indicates that the reaction is not as specifically characteristic of epinephrine as is desirable. This fact might in some cases afford a reasonable doubt as to whether the result obtained with a given commercial sample was due wholly to the contained epinephrine. Consequently for samples of unknown origin a confirmatory test for the genuineness of the material would be necessary in order that unreserved confidence might be placed in the results obtained by means of the phosphotungstic acid reagent.

Disadvantages of less importance are that the blue color fades quite rapidly, therefore necessitating very prompt comparisons of the standards and unknowns. Also that the standard uric acid solution is stable for only a few days. In the case of several standards made by me on succeeding days and then compared simultaneously with each other, rather irregular differences in the developed colors were noted. It is not certain, however, whether these were due to the differences in rate of deterioration of the solutions or to slight errors in measuring the 1 cc. portion to which the phosphotungstic acid reagent is added for the color development.

Considering the very small aliquot portion of the sample required for supplying enough solution for colorimetric estimation, it would appear that the amount of suprarenal used for the epinephrine determination could be very materially reduced and possibly some steps of the method shortened or eliminated.

Finally, attention may be called to the gradual separation of



a crystalline precipitate which often appears in the colored solutions before the comparisons in the colorimeter have been completed. Only one sample of phosphotungstic acid was, however, available for my experiments and it may be that this more or less disturbing precipitate resulted from an impurity in this sample and would not be observed with others.

*Gold chloride reagent.* In the paper of Gautier<sup>6</sup> it is stated that using a 0.33 per cent aqueous gold chloride solution, one drop added to 10 cc. of a 1 : 500,000 solution of epinephrine gives a rose-violet coloration without warming; two drops added to a 1 : 100,000 solution yield a violet coloration and 30 drops added to a 1 : 10000 solution give a beautiful red color. The significance of these observations was not apparent to me until my experiments with a pure epinephrine solution showed that the maximum color production requires that a definite ratio be maintained between the amount of gold chloride and epinephrine present. Experiments on which this conclusion is based were made both by adding increasing amounts of gold chloride solution to given amounts of 1 : 100,000 epinephrine solution and also the same amount of gold chloride reagent to varying dilutions of epinephrine. Thus:

1:100,000 EPINEPHRINE	H <sub>2</sub> O	GOLD CHLORIDE REAGENT		APPEARANCE OF SOLUTION
		Per cent conc.	cc. used	
cc.	cc.			
10	0	0.033	0.1	Very faint pink.
10	0	0.033	0.5	Pinkish color.
10	0	0.033	1.0	More intense pink color than preceding; bluish-pink by transmitted light.
10	0	0.33	0.5	Cloudy opalescence by reflected, bluish by transmitted light.
10	0	0.33	1.0	Cloudy opalescence, brick-red by reflected, blue by transmitted light.
5	0	0.33	5.0	Yellow of the gold chloride practically un- changed.
1	9	0.033	1.0	Trace bluish-pink.
2	8	0.033	1.0	Slightly more color than preceding.
5	5	0.033	1.0	Much more intense than preceding.
10	0	0.033	1.0	About twice as intense as preceding.

<sup>6</sup> *Loc. cit.*

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It is, therefore, seen that if too little gold chloride is used the full color is not produced and if too much is added the solution takes on an opalescent appearance and is characterized by being a pinkish, reddish or yellowish tint by reflected and a bluish color by transmitted light. With a very large excess of gold chloride no epinephrine color is developed at all. The following experiment was made to ascertain the exact ratio of reagent required for the maximum production of color:

EPINEPHRINE SOLUTION 1:100,000	H <sub>2</sub> O	0.033 PER CENT GOLD CHLORIDE SOLUTION	RELATIVE INTENSITIES OF PINK COLOR PRODUCED
cc.	cc.	cc.	
1.0	8.5	0.5	1
1.0	8	1.0	2
1.0	7	2.0	5
1.0	6	3.0	5 (slightly bluish tinge)
1.0	4	5.0	4 (bluish)

A number of experiments were made with other concentrations of epinephrine and it was found that the maximum color was always produced with approximately the same ratio of gold chloride as found in the above experiment. The actual amount of epinephrine present was known since the solution had been prepared by weighing out accurately the carefully purified active principle; the amount of gold in the reagent was also determined carefully and it was found that the ratio of epinephrine to gold in the solution of maximum color was: 0.000010 gram epinephrine to 0.000034 gram gold or, calculated to the molecular basis, 1 gram mol. epinephrine to 3.14 gram atoms Au.

The shade of pink color produced with gold chloride matches very closely that developed by means of potassium iodate as previously described.<sup>7</sup> In regard to the actual intensity of the developed color it was found on comparing the tubes in which the maximum color had been produced with the permanent standards made in connection with the iodate method referred to above, that for a given amount of epinephrine (at least at great dilutions), approximately five times as much color is produced with the gold reagent as with potassium iodate. The observation of

<sup>7</sup> Hale and Seidell: *Amer. Journ. of Pharm.*, lxxxiii, p. 551, 1911.

Gautier in regard to the exceptional sensibility of the gold chloride reaction is, therefore, confirmed by the present experiments.

From the quantitative standpoint, however, since relatively small differences in the ratio of gold to epinephrine were found to make quite appreciable differences in the intensity of color produced, it is evident that an accurate method based on the use of gold chloride must provide steps to first show the approximate amount of epinephrine present. Although this difficulty might not be insurmountable, it seems inadvisable to make further experiments with this reagent until others, which appear not to be incumbered by such limitations, have been studied.

*Manganese dioxide reagent.* A consideration of many of the reagents so far proposed for the colorimetric detection of epinephrine shows that they are, for the most part, characterized by their relatively mild oxidizing power. The pinkish colors produced resemble each other fairly closely, therefore, indicating that a similar reaction is set up in each case. As has been pointed out for the potassium iodate reaction, the pink color is more or less modified by the yellowish extractive material derived from the sample during the boiling required to develop the pink epinephrine color.<sup>8</sup> The intensity of the pink is not sufficient to overcome the effect of the yellow and therefore disturbing variations occur with different samples. In the case of the gold chloride reagent, although a greater intensity of epinephrine color is obtained and this might be sufficient to offset the extractive colors, the necessity for controlling the relative amount of reagent prevents its satisfactory use. It would, therefore, appear that a reagent is needed which can be used in reasonably variable excess

<sup>8</sup> An experiment was made which confirms the assumption that it is the required boiling which so seriously modifies the shade of the developed color. Thus, two 0.010 gram portions of each of four samples of glands were mixed with 10 cc. volumes of water, one set heated to boiling for a few minutes and the other not; the eight mixtures were filtered after two hours. Those which had been heated showed, tube for tube, an unmistakably more brownish appearance than those which had not been heated. Although there was a just perceptible difference in the amount of coloring matter in the unheated tubes, very marked differences were apparent between the four heated tubes, thus showing how two samples with equivalent amounts of epinephrine could easily yield quite different shades of the pink epinephrine color.

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and will develop the epinephrine color without the aid of heat. Of those producing the characteristic pink color which have so far been brought to my attention none appear to fulfil the above-named requirements. In looking about for such a reagent I tried among others, solid manganese dioxide and found that by simply shaking together in a test tube water and a small pinch each of desiccated suprarenal glands and powdered manganese dioxide that the characteristic pinkish epinephrine color slowly develops without the aid of either acid or heat. The maximum intensity appeared to be reached in less than an hour and in common with the pink colors developed by means of mild oxidizing agents, fading proceeded at an exceedingly slow rate. A number of difficulties were of course encountered in adapting this reagent to the satisfactory quantitative determination of epinephrine in desiccated suprarenal glands and the succeeding steps in the development of the method will, therefore, be described in detail.

The first point to be decided, was the optimum amount of manganese dioxide to use. Experiments were made both with pure epinephrine solution and gland samples, and it was found that for 10 cc. of the former containing approximately 1 part per 200,000, 0.0005 gram of  $\text{MnO}_2$  developed slightly less color than 0.001 gram, and that 0.010 gram gave no more color than 0.001 gram. In the case of the desiccated glands it was found that for 0.005 gram of sample plus 10 cc. of water, appreciably less color was obtained with 0.0005 than with 0.002 gram of  $\text{MnO}_2$ , but that 0.01 gram did not increase the color. In both cases it was found that more than 0.10 gram of  $\text{MnO}_2$  diminished the amount of color produced. From these experiments it was concluded that, for 10 cc. portions of epinephrine or gland solution containing up to 1 part per 50,000, 0.005 gram of  $\text{MnO}_2$  would develop the maximum color.

The question of the effect of the presence of hydrochloric acid was studied with results showing that excess of free acid causes a slight diminution of color. The difference caused by 0.1 to 1.0 cc. of 0.1 N HCl per 10 cc. of solution, containing approximately 1 : 300,000 of epinephrine, is, however, barely perceptible, but more acid causes a distinct diminution in color.

The time required for maximum development of color was found to be about one-half hour, but in order to be on the safe side one

hour was always allowed before filtration of the solution preparatory to comparison against the standards. After filtration a just perceptible fading occurs after twenty-four hours' standing.

As in the case of the potassium iodate method annoying differences in the shades of color produced by the reagent in pure epinephrine solutions and the aqueous extracts of the gland samples were observed. This, of course, prevented satisfactory comparisons between the epinephrine standards and the gland samples and left as the only improvement of the new method over the old, the elimination of the heating step and the addition of a definite amount of acid. It was recognized that some way of overcoming this difficulty would have to be found. The first experiments were directed toward procedures for modifying or reducing the amount of foreign color in the gland solutions. These, however, led to negative results and will only be mentioned briefly. Thus, by filtering out the gland sample before adding the reagent, slightly cloudy solutions as dissimilar from the epinephrine standards as those from which the sample was not previously filtered, were obtained. Efforts to develop the color in much more concentrated solutions and dilute to the proper color intensity showed that no advantage was to be gained in this way. The amount of color produced appeared to be independent of the concentration of the active principle at the period of the development of the color.

Since it did not appear feasible to modify the tint of color yielded by the gland samples to make it conform to that obtained from pure epinephrine, it was decided to attempt the reverse procedure, namely, to modify the tint obtained from epinephrine to correspond with that yielded by the desiccated glands. The obvious way to do this is of course to add epinephrine-free gland powder to the solution of the pure active principle, and develop the color exactly as is done with glands of ordinary activity. Preliminary experiments showed this plan to be entirely feasible and a series of color standards were therefore made as follows from epinephrine solution (prepared from the purified principle) and suprarenal sample No. 370, which previous physiological experiments and determinations by the iodate method had shown to be practically free of epinephrine.

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TUBE NO.	1:100,000 EPINEPHRINE SOLUTION	H <sub>2</sub> O	SAMPLE NO. 370	MnO <sub>2</sub>	TIME BEFORE FILTRATION	GRAM EPINEPHRINE PER 10 CC. EQUIVALENT TO COLOR INTENSITY OBTAINED
	cc.	cc.	gram	gram	hour	
a	1.0	9.0	0.010	0.005	1	0.00001
b	2.0	8.0	0.010	0.005	1	0.00002
c	3.0	7.0	0.010	0.005	1	0.00003
d	4.0	6.0	0.010	0.005	1	0.00004
e	6.0	4.0	0.010	0.005	1	0.00006
f	8.0	2.0	0.010	0.005	1	0.00008
g	10.0	0.0	0.010	0.005	1	0.00010

On comparing the tint of color obtained by adding the MnO<sub>2</sub> reagent to various suprarenal samples with the tubes of the above scale the agreement between the shade of color developed in the samples and in the standards was all that could be desired. No difficulty whatever was experienced in locating exactly the position of the sample tube in the scale of standard tubes. The determinations made upon the set of suprarenal samples by this method are given in table II. In each determination 0.01 gram of sample, 0.005 gram of MnO<sub>2</sub> and 10 cc. of water were taken, and the mixture filtered into a test tube at the end of one hour.

It may be mentioned here that for all the experiments described in this paper, except those upon the phosphotungstic acid method, test tubes of diameter and length of approximately  $2 \times 17$  cm. were used. The 10 cc. of solution chosen for all determinations gave a depth of about 4 cm. and the comparative intensities of colors were estimated by observing the tubes from the side, holding them in pairs or threes over a white sheet of paper. Of course any convenient size of test tube may be chosen, but it is important that only those of approximately the same diameter be used in a given series of determinations.

The results obtained by this method are seen to be, with the exception of several of the samples of low activity, higher than found by means of the phosphotungstic acid reagent. In the case of the first eight samples they approach fairly closely the results obtained by the physiological assay as reported in our previous paper. It cannot, therefore, well be claimed that the failure to use hydrochloric acid and heat has resulted in incomplete extrac-

TABLE II.

*Percentage of epinephrine in commercial desiccated suprarenal glands as determined by the manganese dioxide method.*

SAMPLE NO.	SOURCE	DEVELOPED COLOR = THAT OF STANDARD TUBE NO.	CALC. PER CENT EPINEPHRINE
362	Sheep	f	0.8
363	Sheep	f	0.8
365	Sheep	g	1.0
366	Sheep	b	0.2
367	Sheep	f	0.8
368	Sheep	b-c	0.25
369	Sheep	d	0.4
370	Sheep	less than a	0.0
416	Sheep	d	0.4
417	Beef	diluted $\frac{1}{2}$ = f	1.6
418	Hog	c-d	0.35
572	Beef	diluted $\frac{1}{2}$ = e	1.2
573	Beef	f	0.8
574	Beef	diluted $\frac{1}{2}$ = e-f	1.4
575	Hog	c	0.3
576	Hog	a	0.1
577	Hog	c-d	0.35
578	Sheep	d-e	0.5
579	Sheep	a-b	0.15
580	Sheep	d-e	0.5

The order of decreasing intensity of color in the samples as determined by comparison with each other was as follows, the per cent found above by comparison with standards being given in parentheses.

365	(1.0)	$\frac{1}{2}$ (572)	(0.6)	368	(0.25)
362	}	578	(0.5)	575	(0.3)
363		580		366	(0.2)
373	(0.8)	369	(0.4)	579	(0.15)
367		416		576	(0.1)
$\frac{1}{2}$ (417)	(0.7)	577	(0.35)	370	(0.0)
$\frac{1}{2}$ (574)		418			

tion of the epinephrine from the desiccated glands. This appears of particular interest since it is usually considered necessary to digest with dilute acid in order to obtain the full activity of the glands. It, therefore, appeared of interest to test the reaction of aqueous extracts of the glands. This was done by violently shaking a few tenths of a gram of each of several samples in a test

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tube with a few cubic centimeters of water and immediately adding blue litmus paper. A distinct red color was obtained in all cases.

There is one point in connection with the proposed new method which was at first thought to limit its general use, and that is the required epinephrine-free desiccated suprarenals for preparing the standards. It is possible that such material is not of frequent occurrence and a substitute or at least a method for its preparation appears necessary. A simple means for destroying the contained epinephrine in a given sample can no doubt be found, but an experiment made with desiccated thyroid glands showed that this product could readily be substituted for the inactive suprarenals and methods for destroying the active principle in suprarenal glands were, therefore, not sought. The shade and intensities of the developed color corresponded closely with the standards described above and it is evident that perfectly satisfactory results can be obtained with standards made from pure epinephrine solutions and desiccated thyroid gland.

A more serious handicap, however, to the present method, as so far described, is that the costly and difficultly purified epinephrine must be available as the ultimate basis of the determinations. It was, therefore, recognized as necessary that artificial color standards which can readily be reproduced by different analysts must be devised. Experiments along this line were, therefore, next undertaken.

*Artificial permanent color standards for the manganese dioxide method.* As in the case of the color developed with potassium iodate that obtained by means of manganese dioxide is also a mixture of red and yellow. As mentioned in the previous paper permanent standards composed of mixtures of aqueous solutions of cobalt chloride and of potassium platonic chloride were proposed. In the present case it was found that gold chloride could be substituted for the platinum solution and since a standardized solution was on hand, mixtures of it and cobalt chloride solution were used to match the color developed from pure epinephrine and aqueous extracts of the gland samples.

The concentrations of the cobalt and gold solutions which appeared most convenient were: 2 grams crystalline cobalt chloride,  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , dissolved in water, 1 cc. of concentrated HCl added and the mixture diluted to 100 cc. The approximately 0.3 per



cent gold chloride solution used for the previously described experiments was analyzed by evaporating 40 cc., to which a small amount of sugar was added, to dryness, igniting, and weighing the gold. On the basis of this determination the solution was diluted with water to contain exactly 0.1 gram of Au per 100 cc.

By measuring these two solutions from burettes it was found that a mixture of 10 cc. of the cobalt solution and 3 cc. of the gold solution matched exactly the tint of color developed from 10 cc. of a 1 : 100,000 pure epinephrine solution by means of 0.005 gram manganese dioxide as already described. A series of readings in the Duboscq colorimeter showed that a depth of 25 units of the epinephrine color was of exactly the same intensity as a depth of 20 units of the cobalt plus gold color. Therefore to prepare an artificial standard for estimating the epinephrine in pure aqueous solutions, 10 cc. of cobalt solution + 3 cc. of gold solution + 3.25 cc.  $H_2O$  gives a color of exactly the tint and intensity as that yielded by 1 : 100,000 epinephrine solution.

As has already been pointed out the tint of color obtained from gland samples does not match exactly that obtained from the pure epinephrine, it was, therefore, necessary to prepare a series of artificial color standards which would match exactly the colors of the standard scale prepared as already described from pure epinephrine and inactive desiccated suprarenal gland. It was at first thought that since the differences in tint between the color obtained from the glands and pure epinephrine are due only to the yellowish extractive material obtained from the gland itself, that the simple addition of an amount of gold solution equivalent to this extraneous color, to dilutions of the mixture found above to be equal to the color from 1 : 100,000 epinephrine, would give the desired scale of colors. This proved not to be the case, however, and the reason appears to be that, although each sample of glands may yield nearly the same amount of yellow coloring matter, the amount of pink plus yellow epinephrine color varies and consequently there are obtained mixtures of varying ratios of pink and yellow which do not correspond to the color scale made as outlined above.

It therefore became necessary to determine empirically the amounts of cobalt solution, gold solution and water which would

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exactly match each tube of the standard scale prepared from epinephrine plus inactive gland. This was not a very tedious procedure, however, and no difficulty was experienced in obtaining artificial color standards which could not be distinguished from those of the epinephrine. In order to eliminate individual variations to as great an extent as possible the empirically determined proportions of the cobalt, gold and water were plotted on cross-section paper and from the curves so obtained the following table was constructed.

TUBE NO.	COBALT SOLUTION	GOLD SOLUTION	H <sub>2</sub> O	GRAM EPINEPHRINE + 10 CC. H <sub>2</sub> O + 0.010 GM. INACTIVE SUPRARENAL + 0.005 GM. MnO <sub>2</sub> TO PRODUCE EQUIVALENT COLOR
	cc.	cc.	cc.	
1	1.15	0.70	8.15	0.00001
2	1.85	0.95	7.20	0.00002
3	2.40	1.10	6.50	0.00003
4	2.95	1.25	5.80	0.00004
5	3.50	1.30	5.20	0.00005
6	4.05	1.35	4.60	0.00006
8	5.15	1.55	3.30	0.00008
10	6.30	1.75	1.95	0.00010

The 10 cc. mixtures made according to this table were sealed in carefully selected test tubes and form the permanent standards for the estimation of epinephrine in desiccated suprarenal glands. On the basis of 0.01 gram portions of sample, 0.005 gram MnO<sub>2</sub> and 10 cc. of water the developed colors as compared with the above tubes correspond to 0.1 per cent epinephrine in the case of tube No. 1 and 1 per cent in the case of tube No. 10. Therefore, in estimating the epinephrine in a given sample it is only necessary to weigh out the 0.01 gram, add the MnO<sub>2</sub> and water, shake thoroughly and allow to stand one hour, filter into a test tube of dimensions corresponding as nearly as possible to those of the tubes of the above scale and ascertain by comparison against a white background, with which of the standard tubes the sample color corresponds.

In cases of samples which contain more than 1 per cent of epinephrine the filtered solution may be diluted one-half and read against the scale. Under such conditions, however, the tints

may not correspond exactly since by this procedure only one-half of the yellow coloring matter of the sample is present. The difference in tint is usually, however, insufficient to appreciably affect the reading. For very accurate work, therefore, it is better, with samples of high epinephrine content, to take a proportionally smaller amount than 0.01 gram and make up the difference with epinephrine-free gland powder before adding the  $\text{MnO}_2$  and water for development of the color.

It should be mentioned that the artificial color standards as above described have been repeatedly checked against known amounts of the purified ash-free epinephrine and furthermore determinations have been made upon the twenty samples of commercial desiccated suprarenal glands enumerated in table II, using the artificial standards for comparison with results agreeing satisfactorily with those there shown.

The question as to whether various samples of manganese dioxide would yield equal intensities of color was considered and an experiment was made in which five different samples of the reagent were used. Satisfactory agreement in the intensities of the colors was obtained in all cases. In this connection, however, it should be mentioned that while making some preliminary experiments with the method at my request, Mr. Frederic Fenger of Chicago, Ill. found that certain samples of manganese dioxide labeled as being chemically pure gave low results. So far as I know the manganese dioxide used by me was of the grade sold as technically pure. The label on one of the samples indicated that the material was granular pyrolusite. The exceptional results obtained by Mr. Fenger indicate that possibly certain samples designated as very pure manganese dioxide are not as suitable reagents for the present method as the unpurified product or the finely powdered mineral pyrolusite.

A comparison of the results obtained with the phosphotungstic acid method and with the manganese dioxide method shows that the latter gives, in practically all cases except with the samples of low activity, higher results than the former. In fact, if the results by the manganese dioxide method are assumed to be correct, then the phosphotungstic acid method apparently gives low results with the high samples and high results with the samples of low epinephrine content. The question as to which method

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will eventually be found to give results approaching nearest those found by physiological assay cannot be predicted at present.

### SUMMARY.

Attention is called to the defects of the previously proposed potassium iodate method for commercial suprarenal glands and explanations of the sources of error in it are given.

The phosphotungstic acid method of Folin and Denis has been applied to a series of desiccated suprarenals and, although it possesses many excellent features, certain disadvantages which apparently render it unsuitable for the standardization of commercial glands, are pointed out.

The gold chloride reagent was found to yield a maximum color only when a definite ratio was maintained between the amount of epinephrine and of gold, thus seriously restricting the practical quantitative application of this reagent.

The new method proposed in the present paper consists in the use of manganese dioxide as the reagent for developing a color with aqueous epinephrine solutions or suprarenal gland extracts. The color so developed is estimated by comparison with artificial color standards made by mixing cobalt chloride, gold chloride and water.

## THE NATURE OF THE DEPRESSOR SUBSTANCE OF THE DOG'S URINE AND TISSUES.

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The normal urine,<sup>1</sup> the kidney<sup>2</sup> and the pancreas<sup>3</sup> of the dog have been shown by one of us to contain a depressor substance, the action of which can be strikingly demonstrated in kymographic tracing taken after intravenous injection. A number of investigators have studied the effect of the depressor organ extracts from the physiological point of view, and have attempted to bring the characteristic action of these extracts into relation with peptone intoxication and anaphylactic shock, but without success. Recently, Whipple<sup>4</sup> and his associates have shown that the contents of an obstructed duodenal loop (dog) contain a powerful depressor substance very similar in action to that of the urine and kidney of the dog.<sup>5</sup> This observation of Whipple, in view of the studies of Dale and Laidlaw<sup>6</sup> of the effects on the blood pressure of  $\beta$ -iminazolyethylamine produced from histidine when carbon dioxide is split off (Ackermann),<sup>7</sup> which effect is similar in every way to that of the urine and organ extracts of the dog, and again the

<sup>1</sup> Pearce: *Journ. of Exp. Med.*, xii, p. 128, 1910.

<sup>2</sup> Pearce: *Ibid.*, xi, p. 430, 1909.

<sup>3</sup> Eisenbrey and Pearce: *Proc. Soc. of Exp. Biol. and Med.*, viii, p. 93, 1911.

<sup>4</sup> Whipple, Stone and Bernheim: *Journ. of Exp. Med.*, xvii, pp. 286, 307, 1913.

<sup>5</sup> Compare Pearce and Eisenbrey: *Amer. Journ. of Physiol.*, xxvi, p. 26, 1910.

<sup>6</sup> Dale and Laidlaw: *Journ. of Physiol.*, xli, p. 318, 1910; xliii, p. 182, 1911.

<sup>7</sup> Ackermann: *Zeitschr. f. physiol. Chem.*, lxxv, p. 504, 1910.

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work of Mellanby and Twort<sup>8</sup> and Berthelot and Bertrand<sup>9</sup> on the isolation from the intestine of a bacillus capable of producing  $\beta$ -iminazolyethylamine from histidine, suggested that the substance responsible for the fall of blood pressure in peptone intoxication and anaphylactic shock<sup>10</sup> and following the injection of urine and organ extracts (Popielski's "vasodilatin") and Whipple's duodenal loop contents might be one and the same substance. With this possibility in view attempts were made to isolate it or the substance to which it is attached, from dog's urine, pancreas and duodenal loops by the methods of Kutscher and Lohmann and of Engeland. Our experiences have been uniformly negative, and since the results indicate the definite inadaptability of our present methods for the chemical isolation of such substances, presumably bases, we feel that record should be made of our negative results.

Our findings in brief are as follows, being the results of work with the collected urines of three dogs, the collected fluids of three isolated loops of small intestine and of the autolyzed pancreas of ten dogs, toluol or chloroform or both being employed as preservative in all cases. In each case the presence in the original fluid of a strongly depressor substance was first determined by kymographic tracings, following intravenous introduction. Whenever such solutions are carefully precipitated by phosphotungstic acid and hydrochloric or sulphuric acid, with great care in avoidance of excess of reagents, especially of acids, the depressor action remains in the precipitate, and may be recovered therefrom after removal of the phosphotungstic acid. If the precipitation be done however with excess of acid, as commonly advised, the depressor action is destroyed, and is not to be recovered from precipitate or filtrate. When isolation is attempted by means of silver, as in the method of Kutscher and Lohmann, the depressor action is invariably lost; the substance causing it is not to be recovered from precipitate or filtrate. When the solution is submitted to the method of Engeland, the depressor action is likewise lost, as before.

<sup>8</sup> Mellanby and Twort: *Journ. of Physiol.*, xlv, p. 53, 1912.

<sup>9</sup> Berthelot and Bertrand: *Compt. rend. de l'Acad. des Sci.*, cliv, p. 1643, 1912.

<sup>10</sup> Compare Modrakowski: *Arch. f. exp. Path. u. Pharm.*, lxi, p. 67, 1912.

Working with the extract of autolyzed pancreas, we have been able to show that the depressor substance persists after complete removal of proteins (above and including peptone) by tannic acid, *i.e.*, the depressor action remains in the final filtrate. It is therefore not related to the peptone or other protein. On attempting to isolate the substance carrying the activity from this protein-free solution by the method of Kutscher and Lohmann it was lost, as before.

The substance carrying the action is not precipitated by silver nitrate in the absence of barium hydrate; precipitated in the presence of barium hydrate, it is destroyed. Obviously, slight excesses of either acid or alkali operate to destroy, *i.e.*, to set up reactions whereby the chemical nature of the hypothetical substance is so altered as to deprive it of depressor or other toxic properties.

$\beta$ -Iminazolyethylamine (the result of bacterial action or in the commercial form, Ergamine) and dimethyl guanidine pass unscathed through the phosphotungstic acid and silver methods of precipitation; they are therefore not concerned with the depressor substances under investigation. Recently Heyde and Vogt<sup>11</sup> have reported experiments tending to suggest that methyl guanidine is the toxic substance in the urine following extensive superficial burns. From our extract of autolyzed pancreas, no methyl guanidine could be isolated.

Throughout this investigation, at certain stages, small fractions of precipitate and filtrate were removed for blood pressure observations. As at times the disappearance of the depressor substance coincided with the advent of a pressor substance, a few observations on this phase of the work may not be out of place.

The fluid from the mucosa and contents of duodenal loop after heating at 60°C. for an hour and the filtrate after boiling caused a fall of pressure, but the bases finally extracted caused a slight rise. The extract of the pancreas after heating gave a decided fall, but the purified bases, a rise in pressure.

On the other hand, the preparations from the urine, both filtrate and precipitate after phosphotungstic acid, carefully purified, were inert.

Control observation with Ergamine (the commercial name under

<sup>11</sup> Heyde and Vogt: *Zeitschr. f. d. ges. exp. Med.*, i, p. 59, 1913.

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which  $\beta$ -iminazolyethylamine is put out by the Burroughs, Wellcome Company) given intravenously show that the fall in pressure due to urine and pancreas and duodenal loop contents is essentially similar. As, however,  $\beta$ -iminazolyethylamine can be recovered by the chemical methods we have used and the depressor substance of animal tissue and fluids cannot, the two cannot be identical.

### SUMMARY.

Attempts to isolate by the methods of Kutscher, Lohmann and Engeland the depressor substance occurring in the urine, pancreas and duodenal loops of the dog, and which have an effect on blood pressure analogous to that of  $\beta$ -iminazolyethylamine, have failed.



## ON THE DERIVATION OF ETHYL ALCOHOL CONTAINED IN THE MUSCLE.

By ALONZO ENGLEBERT TAYLOR.

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Pennsylvania.)*

(Received for publication, June 6, 1913.)

Freshly killed muscle yields on distillation traces of ethyl alcohol. This alcohol may have been derived from bacterial processes within the alimentary tract. To exclude this source, it is not enough to starve the animal prior to its death. The proteins contained in the alimentary juices, the secretion of which continues to a greater or less extent during starvation, undergo cleavage; from the amino-acids sugar may be formed and this sugar then fermented to alcohol. The only correct method of exclusion of the bacterial processes within the alimentary tract consists in complete removal of the tract. The following report contains the details of such an experiment; and the result indicates that traces of ethyl alcohol are contained in freshly killed muscle under circumstances that practically exclude the alimentary tract as the site of formation.

The operation of complete removal of the alimentary tract in the dog is a difficult operation, which has been skilfully and ingeniously accomplished for me by Dr. Max M. Peet, who will elsewhere report upon the operative features of the procedure. For the purpose of the experiment in hand, it was necessary that no anesthetic be employed that contained or could yield any alcohol, aldehyde or ketone groups; that the circulation of the pancreas and the integrity of this gland be maintained; and that the animal should survive the operation long enough to permit of the combustion of any traces of alcohol that might have been absorbed from the intestine prior to the operation. The anesthetic employed was nitrous oxide with oxygen, and the anesthesia, which was very complete, was in every way satisfactory except that

hemorrhage was free. The entire tract was removed, including the lower end of the oesophagus and the rectum. The animal recovered from the shock of the operation, saline infusion having been used to offset the loss by hemorrhage. Urine was drawn by catheter about eight hours after the operation; it contained neither albumin nor sugar, and contained all the usual components, including some indican and ethereal sulphates, presumably absorbed prior to the operation. The dog was killed eighteen hours after the operation, since death was foreseen to be inevitable within a short time, and I wished to permit no opportunity for post-mortem changes to set in. The animal was hurriedly dissected and a little over 3 kilos of muscle were placed in two large distillation flasks with three times the weight of distilled water, and subjected to distillation. Particular attention was paid to the cooling of the condensers, which were very long and effective, and the receiving flasks were surrounded by ice and fitted with traps. The distillations were continued until two-thirds of the fluid was distilled over. The distillate was redistilled in the same manner, and the distillations repeated until the final distillate was reduced to 25 cc. In one distillation the solution was acidulated with hydrochloric acid. The final distillate gave the iodoform reaction, and without wasting the material on a series of more or less indefinite tests, the entire solution was subjected to Buchner's<sup>1</sup> test, the formation of the ethyl ester of *p*-nitrobenzoic acid, by heating with *p*-nitrobenzoyl chloride. A goodly crystallization followed, the collected crystals (weighing 0.3 gram) were twice recrystallized as suggested by Buchner, and the final yield identified by the melting point and the content of nitrogen. The crystals melted at 57.4°. The known melting point of the crystals of this ester is 57°. Two analyses of 100 mgm. of the ester yielded 7.02 and 7.10 mgm. of nitrogen respectively; the calculated percentage is 7.18.

Have we the right to assume that any traces of ethyl alcohol that were contained in the muscle of a starving dog as the result of resorption from the intestine would have been completely burned within eighteen hours, and that the traces found in this experiment may therefore be regarded as derived from the metab-

<sup>1</sup> Buchner: *Berichte*, xxxviii, I, p. 624, 1905.

olism? When one recalls the velocity of oxidation of alcohol it seems fairly certain that the alcohol found could not have been derived from the intestine. The dog, while starving for one day prior to the operation, was in good nutrition; and samples of muscle tested for glycogen gave good reactions.

From what source could this ethyl alcohol have been derived? It seems most natural to relate it to the metabolism of carbohydrate and to infer that it has been derived from lactic acid, just as in yeast fermentation lactic acid is split into ethyl alcohol and carbon dioxide. If this be correct, one further step in the reactions of combustion of glucose will have been made experimentally clear. Years ago Nef and Buchner suggested that methyl glyoxal might be an intermediary stage in the conversion of glucose into lactic acid. This suggestion has been experimentally verified in recent investigations of Dakin.<sup>2</sup> In a general sense, as once suggested by Bach, the train of reactions involved in the oxidation of glucose might run something like the following, the methyl glyoxal being inserted: glucose  $\rightarrow$  methyl glyoxal  $\rightarrow$  lactic acid  $\rightarrow$  ethyl alcohol  $\rightarrow$  acetic acid  $\rightarrow$  formic acid  $\rightarrow$  carbon dioxide and water, the end products being set free at several stages. Possibly methane might be a stage between acetic acid and formic acid. Dakin<sup>3</sup> has recently shown definitely that formic acid is a normal constituent of urine. The whole scheme, while in part entirely hypothetical and in some respects outside the line of usual interpretation, is harmonious with many known facts in the metabolism of plants and bacteria.

It is obvious that animals deprived of the entire alimentary tract offer opportunities for research in many directions that promise to yield information of decisive value in the determination of different problems. From our present experience with two dogs, we believe that, done with expedition, without extreme shock and hemorrhage, these animals would live for days. In a certain sense, such a dog, with a biliary fistula, would be in much the same position as the animal in complete starvation, except that the organism would be free of the products of bacterial life and of bacterial activity upon the alimentary juices. In such an animal,

<sup>2</sup> Dakin: this *Journal*, xiv, pp. 155, 423, 555, 1913.

<sup>3</sup> Dakin: *ibid.*, xiv, p. 341, 1913.

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for example, one could determine whether there be an endogenous, metabolic fraction of aromatic bodies—phenol, indol and benzoic acid. With the exclusion of the bile, one could investigate the nature of the metabolic pigment of the urine. One could determine whether the ferments of the blood and urine are derived from the alimentary tract. We hope at no distant date to report upon such and other investigations.

# ON THE NATURE OF THE SUGARS FOUND IN THE TUBERS OF ARROWHEAD.

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(Received for publication, June 6, 1913.)

The present paper embodies the result of our study on the nature of the sugars found in the arrowhead tubers (*Sagittaria sagittifolia forma sinensis*), and forms a part of the investigations on the sugars contained in the underground reserve organs of plants, now being conducted in our laboratory.

The arrowhead tuber or Kuwai was selected as the first material to be studied. A search of the literature on *Sagittaria sagittifolia forma sinensis* failed to show the result of special investigations on the composition of its tubers, beyond a brief article by Kellner<sup>1</sup> on their general composition. Consequently, at the beginning of this investigation we have undertaken to test the carbohydrates of the arrowhead tubers and obtained the following result:

Water.....	per cent 78.16
<i>In water-free substance.</i>	
Starch.....	55.32
Dextrin .....	1.75
Reducing sugars.....	0.67 (as glucose)
Non-reducing sugars.....	5.54 (as sucrose)
Mucic acid producing substance by oxidation....	1.43 (as galactose)
Insoluble in 95 per cent alcohol.....	0.69 (as galactose)
Soluble in 95 per cent alcohol.....	0.74 (as galactose)
Pentosane (including methyl pentosane).....	1.83

As has been shown in the above table, of the carbohydrate constituents, starch is a prominent substance, its amount attaining 55.32 per cent of the dry matter. Sugars are also present in no

<sup>1</sup> König: *Chemie der menschlichen Nahrungs- u. Genussmittel*, Berlin, i, p. 705 1903

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slight quantity, reaching the amount of 6.21 per cent of the dry matter, and they form an important part of the carbohydrate constituents.

To determine the exact nature of the sugars of the arrowhead tubers, the following investigation was undertaken.

### *1. Preparation of the syrup.*

The arrowhead tubers were peeled of their rind and finely chopped. The chopped parts were allowed to dry in the air, requiring about two weeks to dry them to such a state that they could be ground easily and finely pulverized.

The preparation of the syrup was begun by extracting 100 grams of the finely pulverized material in a Soxhlet apparatus with ether. The residue so obtained, which was freed from oil, after evaporating the ether was placed in a 750 cc. flask fitted with inverted condenser and treated daily with 300 cc. of 95 per cent alcohol heated in a boiling water bath. The extract was at first of a deep yellow color, but it gradually became lighter from day to day. It had a sweetish taste and was nearly neutral to litmus during the entire time of extraction. One week was required to remove the last traces of sugars. The combined extracts were filtered to remove the sediment which was formed on standing and the filtrate was evaporated to a small volume in a partial vacuum. The concentrated liquor was again extracted many times with a small quantity of absolute alcohol, until no more residue was left after the evaporation of the alcohol. The residue (I) so obtained was preserved for later investigation. The extracts were concentrated to a syrupy condition in a partial vacuum and allowed to dry over sulphuric acid. The preparation was repeated about ten times to get a sufficient quantity for investigation.

### *2. Experiments with the syrup.*

*A. Qualitative tests.* The syrup obtained above, gave the following qualitative reactions:

1. It had a very sweet taste.
2. It reduced Fehling's solution weakly; after inversion with hydrochloric acid, the reducing power was very much enhanced, showing that the non-reducing sugars were present in abundance.

3. Molisch-Udransky's reaction was positive.
4. It gave a characteristic blood-red color by heating with picric acid and a few drops of caustic soda solution (reaction of Braun for glucose).
5. It gave Seliwanoff's reaction very distinctly.
6. It gave Pinoff's reaction for free fructose with ammonium molybdate and acetic acid.
7. It gave characteristic red color by heating in a boiling water bath for exactly one minute with resorcin and alcohol-sulphuric acid mixture according to Pinoff.
8. It did not show any pentose reaction by the phloroglucin method.
9. Mucic acid was produced upon oxidation with nitric acid of 1.15 sp. gr., in the usual manner.
10. Saccharic acid was detected as acid potassium salt in the oxidized solution separated from the crystals of mucic acid by the usual method.
11. It rotated the plane of polarization toward the right; after inversion it was almost inactive.
12. It produced no characteristic mannose phenyl hydrazone with phenyl hydrazine. When the mixture was warmed in a boiling water bath with acetic acid, a yellowish crystalline osazone was produced. Even after inversion, mannose phenyl hydrazone was not detected.
13. Two drops of the syrup were placed on an object glass and were seeded respectively with a crystal of glucose, fructose, galactose, maltose, sucrose and raffinose. After twenty-four hours, the drop which had been seeded with sucrose showed the formation of new crystals, while the others remained unchanged.

From the above qualitative reactions it is safe to conclude that the syrup contains both reducing and non-reducing sugars and that the presence of glucose, fructose, galactose and sucrose is highly probable. Moreover, it is probable that fructose in a free form is present, because the reaction 6, according to Pinoff, is only produced by free fructose while other sugars which contain the fructose molecule in combination as sucrose do not show the same color reaction.

*B. Isolation of sucrose.* When the syrup was left untouched nearly twenty-four hours, it was found thickly laden with fine crystals. A small amount of 95 per cent alcohol was added to the syrup, mixed, filtered by suction, and washed with absolute alcohol and ether. The sugar thus obtained was slightly yellowish in color, but upon recrystallization from alcohol it became perfectly white and left no ash on ignition. After drying over sulphuric acid in a vacuum, its melting point was determined and found to be 159°C.

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0.5 gram of the dried sugar was dissolved in water and made up into 25 cc., and polarized in 200 mm. tube in a Schmidt and Haensch half shadow polariscope. The rotation was found to be 7.7 on the scale toward the right. The specific rotatory power of this sugar is

$$[\alpha]_D = \frac{7.7 \times 0.346 \times 25}{0.5 \times 2} = + 66.6^\circ \text{ (at } 20^\circ \text{)}$$

The melting point and specific rotatory power indicate that the sugar is sucrose.

*C. Osazone tests.* The mother liquor filtered off from the crystals of sucrose was evaporated again to a syrup. After standing for about two days, a small amount of sucrose crystals was again formed in it. The crystals were removed as in the above experiment, and the filtrate was concentrated once more to a syrup. It did not show any sign of forming new crystals even after one week's standing. An attempt was then made to separate and detect the sugars as osazones.

1. One gram of the syrup, 2 grams of phenyl hydrazine hydrochloride, 3 grams of sodium acetate and 20 cc. of water were mixed and heated in a boiling water bath. After fifteen minutes yellowish crystals were produced. At the end of one hour and a half the crystals were examined under a microscope. No other forms, besides the stellate form of the yellow needle-shaped crystals of phenyl glucosazone, were observed. When cooled, it was filtered and washed with a little water. The yellow crystals thus obtained were recrystallized from 60 per cent alcohol and dried over sulphuric acid in a vacuum. The melting point was determined and found to be  $204^\circ$ , which coincides with that of phenyl glucosazone.

The filtrate from the crystals of phenyl glucosazone was heated and concentrated again in a boiling water bath. This produced a second crop of very fine crystals of brownish-yellow color, and their form was identical with that of phenyl galactosazone prepared from pure galactose in our laboratory. After heating for about an hour, it was filtered and washed with a little water. The crystals were recrystallized and dried over sulphuric acid in a vacuum. The melting point was determined and found to be  $193\text{--}194^\circ$ . The crystalline form and melting point indicate that the osazone is probably phenyl galactosazone.



2. One gram of the syrup was dissolved in 20 cc. of water and inverted with hydrochloric acid in a boiling water bath for about thirty minutes. After it was neutralized with sodium carbonate, 2 grams of phenyl hydrazine hydrochloride and 3 grams of sodium acetate were added and heated in a boiling water bath, exactly in the same manner as described above. In this case, none of the other osazones besides the considerable quantity of both glucosazone and galactosazone were obtained.

The osazone tests which were made to separate and detect the sugars in the syrup failed to give a more favorable result than that of the qualitative reactions as already mentioned. But, as the result of this experiment, the absence of maltose may be inferred, because maltosazone can easily be distinguished from the glucosazone in its crystalline form, though its melting point is almost similar to that of the latter. The formation of galactosazone from the original syrup in this case is noteworthy, since the presence of free galactose in nature, up to the present time, had not been reported except by Lippmann<sup>2</sup> who proved its presence in the hoar-frost-like coating of berry ivy after a sudden night frost in autumn. As to whether the formation of galactosazone from the original syrup is due to the presence of free galactose or to a slight inversion of some non-reducing sugar yielding galactose is not yet decided; and the question remains to be solved in the future.

### 3. Experiments with Residue I.

A. *Qualitative tests.* The residue (I) gave the following qualitative reactions:

1. It had a sweetish taste.
2. It did not reduce the Fehling's solution directly; after inversion with hydrochloric acid, it reduced very strongly showing the presence of non-reducing sugars.
3. It did not give the characteristic reaction of pentose with phloroglucin and hydrochloric acid.
4. Molisch-Udransky's reaction was positive.
5. It did not give the characteristic color reaction of Braun with picric acid and caustic soda.
6. It gave the characteristic fire-red color of ketose with resorcin and hydrochloric acid (Seliwanoff's reaction).

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<sup>2</sup> Lippmann: *Berichte*, xliii, pp. 3611-3612, 1910.

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7. It did not give the characteristic color reaction of free fructose with ammonium molybdate and acetic acid.

8. Upon oxidation with nitric acid of 1.15 sp. gr., mucic acid was produced.

9. From the filtrate of the mucic acid crystals, saccharic acid was separated and detected as acid potassium saccharate by the usual method.

10. It did not produce any crystals with phenyl hydrazine hydrochloride and sodium acetate. When the mixture was warmed in a boiling water bath for about thirty minutes, a few crystals of the yellowish glucosazone were produced. After inversion, the glucosazone and galactosazone were formed in abundant quantity by heating for about fifteen minutes.

11. It rotated the plane of polarization toward the right both before and after inversion; though in the latter case the rotation was reduced.

From the above qualitative tests, it is clear that the residue (I) under examination contains some non-reducing sugars which may yield glucose, galactose or fructose.

*B. Isolation of sucrose.* The residue (I) was dissolved with 95 per cent alcohol and evaporated to a syrup. It did not show any sign of forming crystals of its own accord, even after one week's standing. An attempt was then made to purify the syrup by means of basic lead acetate. The syrup was diluted with a sufficient quantity of water, to which a suitable quantity of basic lead acetate solution was added and the mixture well shaken. The fluid soon became turbid and after standing for a little while, a small amount of flocculent precipitate was formed. After separating the precipitate by filtration, a further quantity of basic lead acetate and ammonia was added to the filtrate, when a large amount of a flocculent white substance was precipitated. The insoluble lead compound was collected on a filter with suction, well washed with water, suspended in water and decomposed by hydrogen sulphide. After decomposition was complete, it was filtered and well washed with water, and then the filtrate was evaporated to a small volume in a partial vacuum. The syrup was next extracted with boiling 95 per cent alcohol and separated into two parts, soluble and insoluble, the former being the larger in quantity. The insoluble part of a slightly dark color was designated as Residue II and preserved for later investigation. The soluble part was again concentrated to a syrup.

The purified syrup did not produce any marked crystals even after standing for about one week. Hence, an attempt was once

more made to clarify the syrup by means of absolute alcohol, i.e., the syrup was extracted many times with a small quantity of absolute alcohol until no more residue was left after evaporation of the alcohol. The insoluble part was reserved (Residue III). The extracts were united and concentrated again to a small bulk.

When the twice purified syrup was left untouched for about twenty-four hours, it was found thickly laden with fine crystals. A small amount of 95 per cent alcohol was then added to the syrup, mixed, filtered with suction and washed with absolute alcohol and ether. The sugar thus obtained was perfectly white in color and left no ash on ignition. It was identified as sucrose by its melting point ( $160^{\circ}$ ) and optical rotation ( $+66.53^{\circ}$ ).

#### 4. Experiment with Residue III.

Qualitative tests applied to Residue III showed almost the same reactions as those of the Residue I. A trial was then made to separate and detect the sugar which contains the galactose group. First, we determined how much mucic acid would be produced from the residue by oxidation as follows: 0.2 gram of the residue was put in a small beaker, to which nitric acid of sp. gr. 1.15 was added, and oxidized in a boiling water bath as in the usual manner. The mucic acid produced was collected on the filter and weighed, 0.0065 gram corresponding to 3.25 per cent.

The specific rotatory power of the residue was found to be  $+83.35^{\circ}$ . If the sugar under question be raffinose, the amount of this sugar corresponding to 0.0065 gram of mucic acid would be 0.087 gram according to Creydt.<sup>3</sup> If we assume that sucrose is present besides raffinose, the quantitative ratio of sucrose and raffinose in Residue III would be 1.3 : 1. Upon this ratio, if we calculate the specific rotatory power of the sample, we will then find the value  $+82.98^{\circ}$  which coincides well with that actually observed.

A small amount of methyl alcohol was added to the total residue (III), well mixed and decanted. This operation was repeated until the bulk of the residue insoluble in methyl alcohol was reduced to about half of its original volume. The combined extracts were

<sup>3</sup> Creydt: *Zeitschr. d. Ver. d. d. Zuckerind.*, xxxvii, p. 153; Lippmann: *Chemie d. Zuckerarten*, Braunschweig, 1904, ii, p. 1652.

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evaporated to a syrup. The syrup did not show any sign of forming crystals even after standing for seven days. Absolute alcohol was then added to the syrup to remove matters soluble in the alcohol, well mixed and decanted. The residue insoluble in absolute alcohol was dried in vacuum over sulphuric acid, and its specific rotatory power was found to be  $+92.27^\circ$ .

$$[\alpha]_D = \frac{0.4 \times 0.346 \times 10}{0.015 \times 1} = +92.27^\circ \text{ (at } 20^\circ\text{)}$$

The residue was again treated with methyl and absolute alcohol to remove impurities. The substance finally obtained was almost tasteless. The specific rotatory power was determined and found to be  $-103.8^\circ$ .

The residue was once more purified in the same manner as before. The specific rotatory power was again determined and found to be constant.

$$[\alpha]_D = \frac{0.3 \times 0.346 \times 10}{0.01 \times 1} = +103.8^\circ \text{ (at } 20^\circ\text{)}$$

Next, we determined the quantity of mucic acid which is produced from the residue by oxidation. 0.0834 gram of the residue and nitric acid of sp. gr. 1.15 were mixed in a small beaker and oxidized in a boiling water bath in the usual way. The mucic acid produced was collected on a filter and weighed, 0.0065 gram corresponding to 7.79 per cent.

According to Tollens,<sup>4</sup> the specific rotatory power of raffinose is  $+103.0^\circ$  or  $+104.0^\circ$  while Lippmann<sup>5</sup> found it to be  $+105.0^\circ$  and  $+104.95^\circ$ . The production of mucic acid from 0.1 and 0.075 gram raffinose according to Creydt<sup>6</sup> is 0.009 and 0.0056 gram corresponding to 9.0 per cent and 7.5 per cent respectively.

Consequently it is concluded that the residue is probably raffinose.

<sup>4</sup> Tollens: *Zeitschr. d. Ver. d. d. Zuckerind.*, xxxv, pp. 31, 591; Lippmann: *Chemie d. Zuckerarten*, Braunschweig, 1904, ii, p. 1636.

<sup>5</sup> Lippmann: *ibid.*, xxv, p. 257; xxxviii, p. 1232; Lippmann: *ibid.*, pp. 1636, 1637.

<sup>6</sup> Creydt: *loc. cit.*

*5. Experiment with Residue II.*

The residue, insoluble in boiling 95 per cent alcohol, was slightly dark colored and had a slightly sweet taste. Upon qualitative tests, the residue gave almost similar reactions to those of Residue III. On similar treatment a substance resembling raffinose with a specific rotation of  $+103.7^\circ$  was obtained. It gave 6 per cent of mucic acid on oxidation with nitric acid.

## SUMMARY.

The reducing sugars of the arrowhead tubers consist of both glucose and fructose. Whether the galactose is present as such or in combination with other hexoses is not settled.

The non-reducing sugars consist of sucrose and a sugar which seems to be raffinose.

The presence of maltose, pentose and mannose, free or combined, is excluded.



## THE ACTION OF RENNIN ON CASEIN.

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Changes produced by the action of rennin in milk and soluble casein have been the subject of many investigations. L. J. van Soest was probably the first to give an explanation of this phenomenon. He believed the power to coagulate milk possessed by the inner tract of the mucous lining of a calf's stomach to be due to the presence of an enzyme which converted some of the lactose in milk into lactic acid, the acid thus formed precipitating the

Hammarsten<sup>2</sup> was the first to show that this coagulation of milk was due to the presence of a soluble ferment which acted upon the casein, producing, as he thought, two substances, insoluble curd, *Käse*, which we call paracasein and a soluble whey which he called whey-protein (*Molkeneiweiss*). He also showed that the change of casein to paracasein was independent of calcium salt concentration, the coagulation being due to the presence of soluble calcium salts.<sup>3</sup>

A large number of papers have been published upon this subject since the early work of Hammarsten.<sup>4</sup> As his explanation of the action of rennin has been generally accepted as correct, most recent investigations have been concerned with the influence of soluble salts upon the coagulation. These investigations

by Liebig: *Ann. de pharm.* (Liebig), xxxi, p. 188, 1839.

Hammarsten: *Maly's Jahresbericht*, 1872, p. 118; 1874, p. 135; 1877, p.

also Arthus and Page: *Arch. de physiol.* (5th series), ii.

An excellent review of the literature with references may be found in Bulletin No. 16 of the Hygienic Laboratory of the Public Health and Marine Service of the United States.

have shown that the soluble salts of calcium, barium and strontium favor or hasten coagulation while salts of ammonium, sodium and potassium retard or inhibit coagulation.

Recently Van Slyke and Bosworth<sup>6</sup> have shown that casein and paracasein are acids having the same percentage composition; that the molecular weight of casein is probably  $8888 \pm$ , while the molecular weight of paracasein is one-half that of casein; that both have a combining equivalent of 1111; that combinations of casein or paracasein with one equivalent of calcium, barium or strontium are insoluble in water while the combinations with one equivalent of ammonium, sodium or potassium are soluble; and that ammonium, sodium or potassium caseinates can be changed by rennin to paracaseinates which are soluble and are precipitated by calcium chloride as calcium paracaseinates.

These facts would seem to indicate three things:

First, that rennin action consists of the hydrolytic splitting of the casein molecule into two similar molecules of paracasein; perhaps in somewhat the same manner that maltose is split into two molecules of dextrose.

Second, that, as a consequence of this cleavage it would seem to be doubtful if Hammarsten's whey-protein could be one of the products of rennin action.

Third, that rennin is not, strictly speaking, a coagulating ferment, the coagulation of paracasein being due to the fact that calcium paracaseinates are less soluble than the calcium caseinates, especially in the presence of soluble salts of calcium, barium or strontium.

This investigation was undertaken as an attempt to determine the truth of these statements. In repeating the work of Hammarsten and others a soluble substance which had not been coagulated by rennin and could not be precipitated by dilute acetic acid was always found in the filtrate. Casein solutions for such investigations have been prepared, as a general rule, by shaking pure casein with an excess of lime water or by grinding with moist calcium carbonate. The casein solutions thus obtained were made neutral to litmus and coagulated by the addition of rennin. The curds were filtered off and the filtrates examined for nitrogen.

<sup>6</sup> Van Slyke and Bosworth: this *Journal*, xiv, pp. 203-236.



Soluble nitrogen was always found, but the amounts were not constant and seemed to have no relation to the amounts of casein or rennin used. In the control experiments, to which no rennin had been added, similar amounts of nitrogen which could not be precipitated by dilute acetic acid were also found.

Caseinate solutions prepared in the manner described, contain basic caseinates, either neutral or alkaline to phenolphthalein. As Robertson<sup>6</sup> has shown that such caseinates in solution undergo an autohydrolysis, the following experiment was carried out in order to determine if this might account for the soluble nitrogen found.

Five grams of casein were dissolved in 250 cc. of  $\frac{N}{50}$  calcium hydroxide in the presence of toluol. After complete solution of the casein portions of the solution were withdrawn at intervals and the casein precipitated by means of dilute acetic acid. The casein was filtered off and the nitrogen in the filtrates determined by the microchemical method devised by Folin.<sup>7</sup> The results are as follows:

	3 hours	15 hours	24 hours
Milligrams of nitrogen in original solution.....	158	158	158
Milligrams of nitrogen not precipitated by rennin....	4.0	10.0	28.8

Results of the same nature were obtained with solutions made by grinding casein with moist calcium carbonate. The extent of this autohydrolysis, temperature being constant, depends upon time. As dry casein goes into solution very slowly and freshly precipitated casein is quite rapidly redissolved the following procedure was adopted in order to circumvent autohydrolysis.

Ten grams of pure dry casein were dissolved in 500 cc. of  $\frac{N}{50}$  calcium hydroxide. The casein was then precipitated by adding about 250 cc. of  $\frac{N}{125}$  acetic acid, the liquid was siphoned off, the casein washed several times with water, placed in a linen bag and squeezed as dry as possible. It was then transferred to a mortar, ground to a paste with a little water, the paste put into

<sup>6</sup> Robertson: this *Journal*, ii, p. 344; see also Osborne: *Journ. of Physiol.*, xxvii, p. 398.

<sup>7</sup> Folin and Farmer: this *Journal*, xi, p. 493. All nitrogen determinations made in this paper were made by this method.

a flask and 150 cc. of water, 75 cc. of lime water and some toluol were added to it. After considerable shaking the lime water became saturated with casein. By this process a solution was obtained containing a calcium caseinate neutral to litmus but acid to phenolphthalein,<sup>8</sup> and containing four equivalents of base. The undissolved casein was removed by centrifuging and filtering. The amount of casein in solution was determined and the solution so diluted that each 50 cc. contained 1 gram of casein. Fifty cc. portions of this solution were withdrawn at intervals and precipitated with acetic acid. The amounts of nitrogen found in the filtrates were as follows:

	30 min- utes	5 hours	12 hours	24 hours
Milligrams of nitrogen in original solution.....	158	158	158	158
Milligrams of nitrogen not precipitated by acetic acid.....	0.07	0.92	1.96	2.00

Casein solutions prepared in this manner gave the following reactions. *They were not coagulated by rennin.* The addition of a few drops of a 10 per cent solution of calcium chloride caused them to curdle;<sup>9</sup> the addition of one drop caused no change but the subsequent addition of rennin produced coagulation. If enough hydrochloric acid was added to change the caseinate to one containing two equivalents of calcium,<sup>10</sup> the addition of rennin caused coagulation. That this coagulation was not due to the calcium chloride formed by the acid was shown by the fact that rennin caused coagulation after all this calcium chloride had been removed by dialysis. In both instances *the coagulation removed all the nitrogen from the solution*, as is shown by the following figures:

Milligrams nitrogen in original solution	Milligrams nitrogen not precipi- tated by rennin
316	0.8
316	0.6
316	0.2±

<sup>8</sup> Robertson: this *Journal*, ii, p. 317; Van Slyke and Bosworth: *ibid.*, xiv, p. 211-225.

<sup>9</sup> Robertson: *ibid.*, ii, p. 381. Robertson believes that the addition of the common Ca ion represses the dissociation of the caseinate and thus causes precipitation.

<sup>10</sup> Van Slyke and Bosworth: *ibid.*, xiv, pp. 211-225.

The behavior of such caseinate solutions towards rennin can be explained by the work of Van Slyke and Bosworth as follows:

A molecule of calcium caseinate containing four equivalents of base is split by rennin into two molecules of paracaseinate, each containing two equivalents of base. Such a paracaseinate is soluble in pure water but insoluble in the presence of more than a trace of a soluble calcium salt. A molecule of calcium caseinate containing two equivalents of base is split by rennin into two molecules of paracaseinate each containing one equivalent of base. Such a paracaseinate is insoluble in pure water.

The small amounts of nitrogen recovered in the filtrates in the experiments given above may be due to autohydrolysis or to proteolysis produced by the pepsin in the rennin extract used, as is indicated by the following experiment.

Into each of several flasks were placed 50 cc. of a casein solution and a little toluol. One-half of the flasks received a few drops each of rennin solution, the others being kept as controls. The contents of the flasks were examined at intervals for autohydrolysis and proteolysis. The nitrogen in the control flasks which was not precipitated by acetic acid was considered as due to autohydrolysis; while in the case of the other flasks the nitrogen not removed, by filtering was considered to be due to autohydrolysis and proteolysis. By subtracting the nitrogen found in the controls from those containing rennin a fair idea as to the extent of the proteolysis might be obtained.

	30 minutes	12 hours
Milligrams of nitrogen in original solution as casein....	158	158
Milligrams of nitrogen in filtrate from rennin flasks....	3.4	18.2
Milligrams of nitrogen in filtrates from control autohydrolysis.....	0.1	2.1
Milligrams of nitrogen due to proteolysis.....	3.3	16.1

Solutions of ammonium, sodium or potassium caseinates containing two or more equivalents of base could not be coagulated by rennin, but the subsequent addition of calcium chloride caused coagulation, the curd being calcium paracaseinate. That sodium caseinate in solution was changed to sodium paracaseinate was shown by the following experiment. Rennin was added to a solu-

tion of sodium caseinate and after a short time acetic acid was added. The precipitate, after being purified and dried, was found to be paracasein.

The conclusions drawn from this investigation are as follows:

A solution of calcium caseinate neutral to litmus and free from all other salts is not curdled by rennin.

A solution of calcium caseinate acid to litmus, which contains two equivalents of base for each molecule of casein, is curdled by rennin.

Solutions of ammonium, sodium or potassium caseinates are not curdled by rennin. In such solution however the casein is changed to paracasein, the paracaseinates of these bases being soluble.

When paracasein is produced from casein by the action of rennin no other substance is formed. Two molecules of paracasein are produced from each molecule of casein as a result of this action.

Rennin is not, strictly speaking, a coagulating ferment; the coagulation being a secondary effect, the result of a change in solubilities.

Rennin action is probably a hydrolytic cleavage and may be considered the first step in the proteolysis of casein. It would follow from this that the action now attributed to rennin may be produced by any proteolytic enzyme. Work along this line is being carried out by the author.

In the light of the results reported in this paper together with those of Van Slyke and Bosworth the retarding action of soluble salts of ammonium, sodium and potassium on the coagulation of milk or casein solutions by rennin may be explained as follows. The addition of salts of these bases to milk or casein solutions results in a double decomposition whereby the calcium caseinate is changed to a caseinate of the base added. These are converted to paracaseinates by rennin, but owing to the fact that all the paracaseinates of these bases are soluble, no coagulation results.

In conclusion I wish to express my appreciation of the interest in this work shown by Dr. L. L. Van Slyke, of the Chemical Laboratory of the New York Agricultural Experiment Station, Geneva, N. Y., and Dr. Otto Folin of the Biochemical Laboratory of the Harvard Medical School, Boston, Mass.

THE FORMATION OF INDOPHENOL AT THE NUCLEAR  
AND PLASMA MEMBRANES OF FROGS' BLOOD  
CORPUSCLES AND ITS ACCELERATION  
BY INDUCTION SHOCKS.

PLATE I.

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(Received for publication, June 10, 1913.)

In my earlier paper on the localization of the formation of colored oxidation products in the cells and tissues of the frog,<sup>1</sup> I described experiments in which dimethyl-*p*-diamino-benzene  $C_6H_4.NH_2.N(CH_3)_2$  was used (instead of the usual unsubstituted compound  $C_6H_4(NH_2)_2$ ) in conjunction with  $\alpha$ -naphthol for the intracellular production of indophenol. A mixture of these two substances forms on oxidation dimethyl indophenol, a deep blue, water-insoluble compound which is deposited within the cells in the form of conspicuous granules; these show in many cases a highly definite distribution; thus in red corpuscles and leucocytes a tendency to deposition at the surface of the nuclear membrane is very constant and pronounced. This appearance is so definite that it seemed at that time to afford strong support to the view that the cell nuclei have a special relation to oxidation processes. The photographs on plate I (figs. 1 and 2) illustrate this condition in corpuscles that have lain for some minutes in a solution of  $\alpha$ -naphthol and dimethyl-*p*-diamino-benzene in physiological salt solution. It will be seen on referring to these photographs that the granules at the boundary of nucleus and cytoplasm in the erythrocytes are larger and more densely massed than elsewhere in the cell.

There is little doubt that the regions where the indophenol is

<sup>1</sup> *Amer. Journ. of Physiol.*, vii, p. 412, 1901.

most densely deposited within these cells correspond to the regions of its most rapid formation, and hence to the regions of most active oxidations. Indophenol granules show an active Brownian movement when freely suspended,—as in a solution of  $\alpha$ -naphthol and *p*-diamino-benzene undergoing spontaneous oxidation; but when the granules are deposited in the interior of the cells little or no movement is perceptible, and under these conditions they must tend to remain in the place of their formation. Direct observation shows that their aggregation at the nuclear membrane of erythrocytes is not the effect of a secondary gathering in this situation, subsequent to formation elsewhere in the cell; the first granules that make their appearance in erythrocytes after placing in the indophenol-forming solution almost always occupy this position, and it is not until later that granules appear elsewhere in the cell. The region adjoining the nuclear surface in erythrocytes appears thus to be a region of relatively rapid oxidation. Evidence that oxidative processes of this kind may show definite localization in cells acquires especial significance in view of Vernon's recent observations, according to which the rate of formation of indophenol is a fair index of the general oxidative power of the tissue;<sup>2</sup> if this is the case, the conditions of formation of this compound in cells probably correspond in the main to those of the other and physiologically important oxidations.

Further study has however convinced me that the nuclear surface is not, in general, necessarily the seat of more active oxidations than the other free surfaces or phase boundaries in cells. The oxidation process appears to be strongly influenced by the conditions at surfaces; adsorption and variations of phase-boundary potentials are probably the chief factors in this influence, as apparently in other forms of catalysis due to surface action. I find that in voluntary muscle cells the indophenol formation shows no evident relation to the scattered superficial nuclei, but appears diffused throughout the cytoplasm. Again, in frozen sections of the frog's brain and spinal cord the distribution of most active indophenol formation does not correspond to the distribution of nuclei in these tissues; the oxidation is, in fact, more active in the white than in the gray matter. These facts,

<sup>2</sup> Vernon: *Journ. of Physiol.*, xlii, p. 402, 1911.

as well as various more general considerations partly discussed below, suggest that the formation of indophenol at the nuclear surface in blood corpuscles is not to be regarded as indicating a specific relation of nuclei to oxidations; it seems more probable that it indicates the existence of a general relation of the cell-surfaces or protoplasmic phase boundaries to oxidations. There are other facts indicating this. Warburg's observations on sea-urchin eggs show that alkali may modify oxidations without penetrating the cell—apparently by altering the state of the general cell-surface or plasma membrane.<sup>3</sup> Nernst's theory of electrical stimulation implies that the rate of oxidation in irritable tissues is increased by varying the state of electrical polarization of the semi-permeable cell surfaces; *i.e.*, increase in oxidation is a very general consequence of electrical stimulation, whose essential condition is alteration of the electrical polarization at such surfaces.

Re-examination of the conditions of indophenol formation in frog's blood corpuscles has confirmed my former observations of a relatively rapid oxidation at the nuclear surface; the appearances indicate also that a second region of relatively rapid oxidation exists near the general outer surface of the cell, or just within the plasma membrane. It is difficult to obtain unequivocal proof of this in the case of erythrocytes, on account of the flattened shape of these cells; but when the cells are slightly crenated and are viewed in face with a high power, the granules of indophenol often show a network-like disposition which apparently corresponds to the folds of the surface; examination with the oil-immersion lens—especially when the cells are viewed edgewise—also often shows a denser distribution of granules just within the cell-surface than in the region intermediate between plasma and nuclear membranes. The region of densest deposition of granules in erythrocytes is, however, always the nuclear surface, and apparently this is the region of most active oxidation in these cells.<sup>4</sup> In leucocytes a relation of oxidation to the general

<sup>3</sup> Warburg: *Zeitschr. f. physiol. Chem.*, lxvi, p. 305, 1910.

<sup>4</sup> Warburg's observations on the rate of oxygen consumption by the red corpuscles of birds show an interesting parallel with those just cited (cf. Warburg: *Zeitschr. f. physiol. Chem.*, lxx, p. 413, 1911; *Münch. med. Wochenschr.*, lviii, p. 289, 1911). These cells show a much higher oxygen consumption, especially when newly formed, than the non-nucleated ery-

cell-surface is more distinct. Indophenol is formed in these cells much more rapidly than in erythrocytes, and in a mixture of  $\alpha$ -naphthol and dimethyl-*p*-diamino-benzene solutions, of the composition given below (page 243), the surface layer of the cytoplasm of large leucocytes, as well as the region adjoining the nucleus, quickly becomes filled with large and conspicuous granules. The presence of numerous granules just within the cell-surface is highly characteristic of leucocytes that have lain for a few minutes in the indophenol-forming solution. Later the whole cytoplasm becomes densely laden and the original inequality of distribution is obscured. In some of the photographs reproduced with this article rows of granules just within the cell-surface are clearly seen (see figs. 4 and 5).

These observations show that a close relation exists between oxidations and certain intracellular surfaces or phase boundaries. It is theoretically improbable that the surfaces of semi-permeable membranes like nuclear or plasma membranes are alone concerned in these processes. The surfaces of other colloidal structures probably play a similar part in cells; thus in muscle cells it is possible that the large surface of contact between fibrils and sarcoplasm forms a region active in oxidations, although there is no direct evidence of this as yet; if so, the high oxidative activity of these cells may in part be accounted for. Increased reaction velocity in polyphasic systems with large surface extent is a frequently observed phenomenon; the catalytic action of colloidal metals has been explained by Bredig and others as an

throcytes of mammals. Alternate freezing and thawing destroys the cytoplasm, but leaves the nuclei intact; cells thus altered show an unimpaired or even increased rate of oxidation. Evidently the general cell-surface is not essential to oxidation in these cells; if however the nuclear surface is the active region in birds' erythrocytes, as in those of frogs, Warburg's results may readily be accounted for. His further observation that salts like calcium, magnesium, and barium chloride influence oxidations *after*, but not before, destruction of the plasma membranes also receives a consistent explanation, since the plasma membranes are normally impermeable to these salts, which accordingly have free access to the nuclear membrane only after the plasma membranes are destroyed. On the other hand, phenyl, methyl, and ethyl urethanes, and hydrocyanic acid, which penetrate the plasma membranes with ease, influence oxidations equally in intact and injured erythrocytes.



instance of such surface action, and this action is very generally referred to increased concentration of the interacting substances at the phase boundaries. It is probable that the electrical condition of these surfaces also constitutes an important factor in the catalytic action.<sup>5</sup>

The importance of surface processes in cell activities has been widely recognized of recent years, especially since the rise of colloidal chemistry and its application to physiological problems. The character of the electrical polarization at surfaces is known to influence profoundly the adsorptions at such surfaces,<sup>6</sup> and any chemical changes dependent on adsorption (as catalyses of the class referred to above) must be correspondingly influenced by the electrical condition of the surfaces. Hence chemical processes occurring in polyphasic systems must in many cases be influenced by changes in the polarization of the surfaces, to a degree which under some conditions may largely determine the general course and velocity of certain reactions; and the possibility that the chemical effects of stimulation may belong in this category thus requires consideration. According to Nernst's theory, changes in the electrical polarization of the plasma membranes form the essential condition of electrical stimulation. The same is almost certainly true of other forms of stimulation, including the normal, since all are accompanied by electrical variations whose characteristics point unmistakably to variations of phase-boundary potentials as their determining condition.<sup>7</sup> The rate of oxidation in voluntary muscle cells is increased

<sup>5</sup> See the communication of S. W. Young: On the Influence of Light on the Electric Potential of Bacterial and Other Suspensions, in the *Proc. of the Soc. for Exp. Biol. and Med.*, x, p. 151, 1913.

<sup>6</sup> Cf. L. Michaëlis in Koranyi and Richter's *Physikalische Chemie und Medizin*, ii, 1908, pp. 347 seq.; Michaëlis and Ehrenreich; *Biochem. Zeitschr.*, x, p. 283, 1908; Höber: *Physikalische Chemie der Zelle und der Gewebe*, 1911, p. 294; Wolfgang Ostwald: Die neuere Entwicklung der Kolloidchemie in *Kolloidchemische Beihefte*, 1912, iv, p. 16. Ostwald distinguishes "electrical adsorption" from adsorption due to lowering of the surface tension at the phase boundary. In general adsorption occurs whenever the energy potential at the phase boundary is lowered by the accumulation of the dissolved substance in this region.

<sup>7</sup> Cf. my paper on the relation of membrane-changes to stimulation and conduction, in *Amer. Journ. of Physiol.*, xxviii, p. 197, 1911.

many times by stimulation; and investigation of the action current of this tissue with the thread galvanometer shows that stimulation is normally associated with a rhythmical electrical variation of a definite rate; this rate varies with temperature according to the chemical temperature coefficient,<sup>8</sup> *i.e.*, shows a general parallelism with the oxidative and other chemical activity of the tissue as influenced by temperature change. An interdependence of some kind is thus indicated, and it seems likely that this interdependence is direct, and that the polarization changes form the immediate condition of the oxidations. The mechanism of intracellular oxidations is still largely obscure, and it is admitted by most biological chemists that the known properties of oxidases cannot account for the character and high velocity of the intracellular oxidations. No enzymes or combinations of enzymes and co-enzymes can accelerate the oxidation of sugar or lactic acid to anything like the degree required. Such facts suggest that the oxidases are not the main factors in intracellular oxidations, but play a merely accessory part; their presence may be favorable to rapid oxidation (by diminishing resistance), but the essential determining conditions appear to be of a quite different kind. Some feature or features of the structural organization of the cell must be fundamentally concerned, since destroying cell structure always greatly diminishes the oxidative activity of the tissue.<sup>9</sup> Now the semi-permeable membranes of irritable tissues constitute the structural elements which are primarily essential to electrical stimulation, and hence to the increase of oxidation which is associated with stimulation. According to Nernst's theory, stimulation involves changes in the electrical polarization of these membranes. Such changes of surface polarization must involve corresponding changes in the polarization of the other polarized surfaces within the cell; *i.e.*, the conditions of equilibrium of the double layers at these intracellular surfaces will be altered by altering the polarization of the general cell-surface, and their state of polarization will undergo corresponding change; hence the normal variations of polarization at the cell-surface accompanying stimulation must involve similar changes at the active surfaces throughout the cell, and it is these polarization changes

<sup>8</sup> Cf. Piper: *Arch. f. (Anat. u.) Physiol.*, 1910, p. 207.

<sup>9</sup> Cf. Fletcher and Hopkins: *Journ. of Physiol.*, xxxv, p. 287, 1907.

—according to the hypothesis which I am presenting—that mainly determine the increase in the rate of oxidation. Similar or related views have been tentatively put forward by a number of investigators,<sup>10</sup> but hitherto little or no experimental evidence in their support has been adduced. The experiments about to be described show that the formation of indophenol in leucocytes—a process largely dependent on surface action as already described—can in fact be markedly accelerated by the passage of induction shocks through a suspension of corpuscles on a slide.

#### EXPERIMENTAL.

In the following experiments the blood corpuscles of the frog have been used. Immediately after shedding, the blood is mixed with a solution consisting of nine volumes  $\frac{M}{8}$  NaCl *plus* one volume  $\frac{M}{8}$  potassium oxalate; this solution prevents clotting and breakdown of leucocytes. The suspension of corpuscles is then mixed with the solution of the indophenol-forming reagents in slightly alkaline physiological salt solution. The solution which I have chiefly used is made (shortly before using) by mixing a saturated solution of  $\alpha$ -naphthol in alkaline isotonic NaCl solution ( $\frac{M}{8}$  NaCl containing  $\frac{M}{100}$   $\text{Na}_2\text{CO}_3$ ) with a 0.5 per cent to 1 per cent solution of Merck's dimethyl-*p*-diamino-benzene in  $\frac{M}{8}$  NaCl. These concentrations are favorable to the rapid formation of indophenol within the cell. With more dilute solutions the formation of indophenol is slower and its acceleration by induction shocks is less evident. Usually equal volumes of these solutions (1 cc. of each) are mixed in a test tube, and the resulting solution is then mixed intimately with a few drops of the suspension of corpuscles either in a watch glass or on a slide. This preparation may be mounted and examined at once or after the desired interval of time has elapsed. Leucocytes left in this solution (with sufficient access of oxygen) become deeply laden with indophenol granules in a few minutes. In erythrocytes the deposition of granules is more gradual; in these cells the first formed granules typically appear at the nuclear surface, as already described. Figures 1 and 2 are photographs of corpuscles taken after remaining for twenty-five and fifty-two minutes respectively

<sup>10</sup> Cf. Warburg, 1910, *loc. cit.*

in a solution of the above composition. By this time the leucocytes are so densely laden with indophenol as to prevent the appearance of mere clumps of granules. The erythrocytes are much less deeply impregnated and the contours of the nuclei are plainly marked by the deposition of granules.

The accelerating influence of induction shocks on this oxidation may be demonstrated as follows. Immediately after mixing with the reagent, as above described, a few drops of the suspension of blood corpuscles are mounted on a specially prepared slide crossed by two fine parallel platinum wires about 2 cm. apart. The slide is already in position on the microscope stage; the wires are connected to the secondary coil of an inductarium (Porter's model) arranged for "tetanizing" currents, and a succession of shocks is passed through the preparation. If a suspension of corpuscles so treated is compared with a control consisting of a portion of the same suspension similarly mounted on a precisely similar slide (so as to have conditions of oxygen supply, etc., as nearly alike as possible), but not subjected to induction shocks, a marked difference soon becomes evident. Within a brief period, varying according to the strength of the shocks and concentration of the solution, the leucocytes in the "stimulated" preparation are seen to be plainly more deeply impregnated with indophenol than those of the control. The contrast is very striking if the conditions of concentration, number of corpuscles,<sup>11</sup> and strength of stimulation are favorable (see plate I, figs. 3-5). The difference is not due to the oxygen liberated at the wires serving as electrodes, for the acceleration of indophenol formation is seen throughout the entire region between the electrodes.

The following protocols of experiments will illustrate the character of these observations.

*November 4, 1911.* The blood of a large frog was mixed with an approximately equal volume of oxalate-containing sodium chloride solution. A drop of this suspension of corpuscles was placed on a slide provided with platinum wires; a drop of the indophenol-forming mixture was placed in

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<sup>11</sup> Too dense a suspension of corpuscles is unfavorable to this demonstration, probably because the oxygen is too rapidly abstracted by the erythrocytes, leaving less available for oxidation in the leucocytes.

contact with the drop of suspension and a cover glass was put in place. Induction shocks were then passed.

In six successive experiments of this kind the leucocytes were found to form indophenol granules more rapidly under this treatment than in the untreated control. In some experiments the contrast at the end of two or three minutes was very striking; the leucocytes near the edge of the cover glass, where most oxygen was available, being then so deeply laden as to appear like masses of granules, while in the control only a moderate deposition of granules had taken place.

Numerous other experiments showed similar results. Usually the suspension of corpuscles and the indophenol-forming mixture were mixed before mounting. The following typical experiments are cited in further illustration.

*November 15, 1911.* The suspension of corpuscles was mixed with the freshly prepared indophenol-forming solution in a watch-glass and then mounted on the slides with platinum wires. Induction shocks from a Porter's inductorium with three Edison primary cells and coils 3 cm. apart were passed through the preparation. Nine experiments were performed under these conditions. The following results are typical.

**EXPERIMENT 3.** At 12.11 the current is started through the preparation. The granules appear rapidly in the leucocytes; at 12.16 the field of the stimulated preparation is full of intensely colored leucocytes; in the control, although many leucocytes are well laden with indophenol, the number of deeply impregnated cells and the degree of loading are much less. By 12.20 the erythrocytes of the stimulated preparation also show a distinctly deeper tint than those of the control.

**EXPERIMENT 4.** Current started at 12.37. The result is similar to the above; in three minutes there is a decided contrast between experiment and control. At 12.43 the erythrocytes also show considerable indophenol; the nuclear membranes appear to be impregnated with the dye, and stand out sharply. Some leucocytes show granules at the nuclear surface, and others show rows of granules just within the cell-surface.

**EXPERIMENT 8.** Conditions and results the same. The contrast between the microscopic fields of experiment and control under a low power is striking; eight minutes after starting the current the field of the stimulated preparation appears dotted at intervals with intensely blue-black dots corresponding to the leucocytes; in the control the leucocytes stand out much less conspicuously.

The above records are sufficient to indicate the general character of these observations. It will be noted that the induction shocks used in the above experiments are of considerable strength. Weaker shocks also accelerate the rate of oxidation in leucocytes but less obviously. It is also noteworthy that the effect on the

erythrocytes is comparatively slight; the oxidative activity of these cells, besides being much less energetic than that of the leucocytes, is less easily influenced by electrical stimulation, although after passing strong shocks for some minutes there is an appreciable increase in the formation of indophenol. The much higher oxidation rate and greater responsiveness of the leucocytes correspond to the more active part which these cells play in the organism; thus it is not surprising to find that their oxidation rate—and hence their general rate of metabolism and output of energy—is more easily altered by changes of condition. Leucocytes, although relatively inert as compared with (*e.g.*) muscle cells, have in fact many of the characteristics of irritable cells. Hamburger has shown that their phagocytic activity may be increased artificially by a variety of substances, including calcium salts and lipoid solvents in low concentration.<sup>12</sup> The conditions under which oxidations are accelerated in these cells are thus probably of the same essential kind as those prevailing in irritable cells in general.

The possibility naturally suggests itself that various chemical processes other than oxidations may be similarly influenced by changes of polarization at the intracellular surfaces. The formation of indophenol is in fact a synthesis involving dehydration, as well as an oxidation, but probably no especial significance attaches to this fact. What seems to be significant is that the chemical process occurs in close relation to the intracellular surfaces, and is influenced by changes in the electrical polarization of these surfaces.<sup>13</sup> It seems likely that conditions of this kind play an important general rôle in cell metabolism. The precise conditions of the above electrochemical effect are largely obscure to me at present. The analogy with photochemical action seems

<sup>12</sup> Hamburger and Hekma: *Biochem. Zeitschr.*, ix, p. 275, 1908; Hamburger, de Haan and Bubanovic: *Archives Néerlandaises des Sciences Exactes et Naturelles*, Ser. III, B., i, p. 1, 1911. Hamburger's recent book: *Physikalisch-chemische Untersuchungen über Phagozyten*, Wiesbaden, J. F. Bergmann, 1912, contains a full account of these and related observations.

<sup>13</sup> The formation of peroxides may possibly be favored by this process, just as the formation of ozone and hydrogen peroxide is favored by the passage of electric discharges through moist air. There is good evidence that peroxides play an important—though it may be a supplementary—rôle in protoplasmic oxidations.

perhaps the most illuminating. In both cases increased displacement, or increased range of movement, of electrons—due to the electro-magnetic effect of the ether waves, or of the variation of surface polarization—and consequent facilitation of the electron transfers that condition the chemical change, are probably the fundamental factors of the action. Further discussion of this matter would, however, be inappropriate in this place.

#### SUMMARY.

1. In frogs' blood corpuscles the formation of indophenol by the intracellular oxidation of a mixture of  $\alpha$ -naphthol and dimethyl-para-diamino-benzene takes place most rapidly in the immediate neighborhood of the nuclear and plasma membranes. The conditions at the surfaces of these structures are thus particularly favorable to rapid oxidations.

2. The passage of induction shocks through a suspension of corpuscles in the indophenol-forming solution accelerates this oxidation; this effect is slight in erythrocytes and well marked in leucocytes.

3. These observations indicate, in general, (a) that the intracellular surfaces or phase boundaries play an active rôle in oxidations in living cells, and (b) that variations in the electrical polarization of these surfaces form an important factor in these oxidations, and especially in the increase of oxidations following stimulation.

## EXPLANATION OF PLATE.

The photographs were made by Dr. H. G. Kribs, of the Zoölogical Department, University of Pennsylvania, to whom I take pleasure in expressing my best thanks. The magnification is about 500 diameters. The exposures were brief (5 to 10 seconds) and were made within one to two minutes after the stimulating current had ceased. The corpuscles shown in these photographs are entirely typical of the conditions throughout the preparation.

Fig. 1. Showing spontaneous formation of indophenol in corpuscles. The cells were photographed after lying undisturbed for twenty-five minutes in the indophenol-forming solution. The densely laden central corpuscle is a leucocyte. The round dots scattered over the field are indophenol granules; the halo about each is due to Brownian movement.

Fig. 2. Corpuscles from a similar "unstimulated" preparation after fifty-two minutes in the solution. The round black mass just above the middle of the figure is a leucocyte. The rows of granules at the nuclear surface in the erythrocytes are typical.

Fig. 3. Stimulated preparation. The corpuscles were placed in the freshly prepared indophenol-forming solution and one-half minute later induction shocks were passed for three minutes through the preparation (mounted on a slide as described in the text. Two Edison primary batteries; Porter's inductorium with coils 2 cm. apart). Exposure was made one minute after current ceased. Four leucocytes are shown in the field; the one at the left is less densely laden than the other three.

Fig. 3a. Control of figure 3. The corpuscles were mounted as in the experiment of figure 3 but not subjected to induction shocks. Exposure was made after remaining for the same length of time (five and one-half minutes) in the solution. Six leucocytes in the field, all showing considerable formation of indophenol, but much less than in the stimulated corpuscles.

Fig. 4. Conditions the same as in the experiment of figure 3. Three deeply laden leucocytes in the field.

Fig. 4a. Control of figure 4 (in the solution for the same length of time, but unstimulated). Two leucocytes seen, showing much less indophenol formation than those of figure 4.

Fig. 5. Conditions like those of the preceding experiments, but shocks passed for only two minutes. Exposure four and one-half minutes after placing in the indophenol-forming solution. Two leucocytes are seen, showing typical conditions.

Fig. 5a. Control of figure 5. In solution for the same length of time before exposure. Two typical leucocytes shown.







## THE DOMINANCE OF ROQUEFORT MOLD IN CHEESE.<sup>1</sup>

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Roquefort and certain related types of cheese contain practically pure cultures of Roquefort mold (*Penicillium roqueforti* Thom). Roquefort in its manufacture is inoculated with this mold prepared in pure culture. Gorgonzola is not. Stilton is not. Yet these latter cheeses contain this mold in fairly pure condition. What factors lead to the dominance of this mold in such cheeses, whether it is put in during the making period or not? Study of large numbers of milk cultures shows clearly that nearly all milk contains spores enough of various species of *Penicillium* as well as other molds to admit of a wide variety of mold colonies in any cheese provided conditions are favorable for such molds to grow. Clearly then there are factors present which favor this species in competition with the many other forms which are initially present in milk and which have been shown to grow equally as readily upon milk and milk products as does Roquefort mold. Preliminary cultural experiments by one of us, together with the work of Clark upon Emmental cheese directed our attention to the gases of the cheese as a probable factor in this dominance.

Clark<sup>2</sup> studied the gases of Emmental cheese as an index to the biological factors concerned in its ripening. This variety of cheese has attracted special attention because of the scientific interest and the economic importance attached to "eye" formation. A cheese having the texture of Swiss is quite impermeable to gases and the gases produced are retained in the cheese near

<sup>1</sup> Published by permission of the Secretary of Agriculture and the Director of the Storrs Agricultural Experiment Station under whose coöperative arrangement the work was done.

<sup>2</sup> Clark: U. S. Dept. of Agric., Bureau of Animal Industry, Bulletin 151, 1912.

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their place of origin. The origin of the gases is therefore the problem of chief interest. A cheese having the texture of Roquefort presents an entirely different problem. *Penicillium roqueforti*, which is probably the chief factor in the ripening of this variety of cheese, is not an anaerobic organism and in order that enough oxygen may diffuse through the cheese mass to sustain the growth of this mold, it is made with as open texture as possible and about forty holes the size of a knitting needle are punched through it by means of the "prickle machine." This investigation was undertaken with the hope of getting some definite information concerning the gases within the cheese and their relation to the growth of *P. roqueforti*.

### *Method of collecting the gas.*

The apparatus used in collecting the gas is shown in fig. 1. The puncturing part of the apparatus consisted of a glass rod *A* which fits snugly into the glass tube *B*. The tube *B* was cut in two about one inch from the lower end. The lower section was sealed fast to *A* and the upper section could be slid up or down. With the two sections of *B* together, the puncturing part of the apparatus was pushed nearly through the cheese. The upper section of *B* was then pulled up into the position shown in the figure, the adjoining section of capillary tubing connected and cock *C* closed. The entire cheese was then coated with paraffine by repeated dipping. The dipping was conducted slowly to prevent heating the interior of the cheese. When the paraffine coat had attained a thickness of about one inch, the entire cheese was imbedded in paraffine nearly at the solidifying temperature. When the cheese was thoroughly cooled the apparatus was connected as shown in the diagram. A calcium chloride tube *D* was placed between the cheese and the gas-collecting tube *G* to intercept any water that might be drawn up with the gas. The collecting tube *G* had a capacity of 230 cc. *E* led to a manometer and *K* to a water suction pump. With the cock *C* closed, the apparatus was exhausted to the limit of the pump which was about 25 mm. Cock *H* was then closed, and when assured that the apparatus was perfectly tight cock *C* was opened and the gas within the cheese allowed to expand into the collecting tube. When the pressure almost or entirely ceased to rise, cock *F* was closed and the collecting tube disconnected. The amount of oxygen and nitrogen from the air remaining in the collecting tube, was determined by exhausting to exactly the same pressure as that at which the gas was collected, and measuring the air by displacement with mercury.

This method of collecting the gas, although simple, appears to give accurate results. The amount of oxygen is no greater than is really to be expected in a cheese in which gaseous diffusion is as free as in Roquefort. The most probable error is due to the

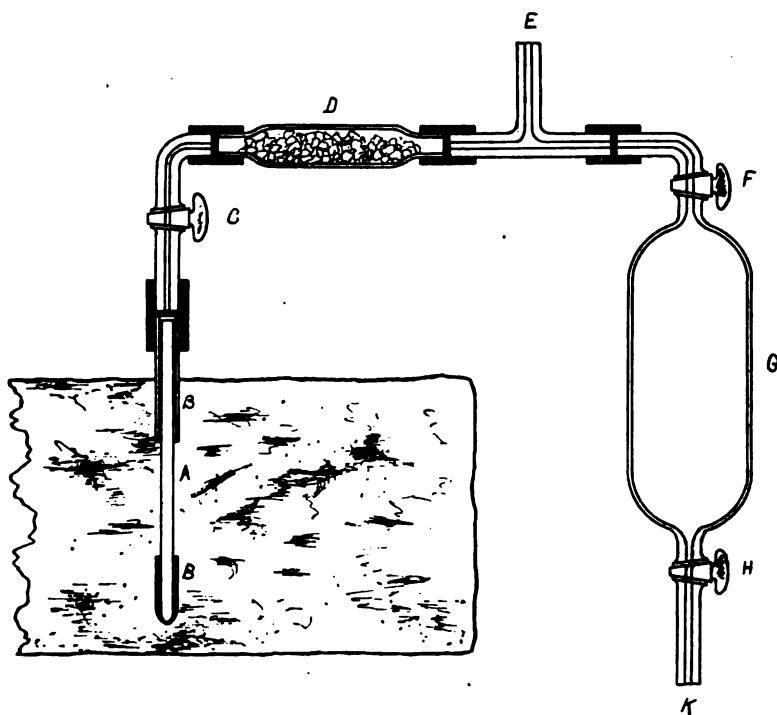


FIG. 1. APPARATUS FOR COLLECTING GAS.

film of air between the cheese and the first layer of paraffine. At least twenty-four hours elapsed between the time the first coat of paraffine was applied and the time the gas was collected. The respiration of the microorganisms on the surface of the cheese and the diffusion of the gases throughout the cheese would greatly reduce this error. The ingenious apparatus devised by Clark for the study of Swiss cheese is not adapted to a cheese having the texture of Roquefort.

The gas was displaced from the collecting tube with mercury, the carbon dioxide determined by absorption over potassium hydroxide and the oxygen by absorption over phosphorus. The residual gases of cheeses 2 and 5 were burned with air in a mercury pipette by means of an electrically heated, platinum spiral. Neither sample showed the presence of hydrocarbons. The gas from cheese 5 gave a contraction which would have corresponded to 1.0 per cent of hydrogen. The residual gas of cheese 3 was

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tested for hydrogen by repeated passing over palladium at 90°C. The gas showed no reduction in volume. The conclusions were therefore drawn that the residual gas consisted chiefly of nitrogen, that it was free from hydrocarbons but may have contained small amounts of hydrogen. It is reported as nitrogen in the following table.

TABLE I.  
*Analyses of the gases of Roquefort cheese.*

BRAND	CC. OF GAS AT 0°C. 760 MM.					PER CENT			REMARKS
	Gas collected	Gas analyzed	CO <sub>2</sub>	O <sub>2</sub>	N <sub>2</sub>	CO <sub>2</sub>	O <sub>2</sub>	N <sub>2</sub>	
1. Louis Rigal	18.21	18.21	5.07	0.44	12.21	27.84	2.42	69.74	Slight growth of mold.
2. Société.....	123.60	44.31	11.19	1.48	31.64	25.25	3.34	71.44	Slight growth of mold.
3. Veritable Roquefort	81.73	81.73	18.07	5.72	57.94	22.11	7.00	70.89	Very ripe and moldy.
4. Experimental cheese	88.25	80.29	32.88	3.64	43.77	40.95	4.53	54.52	Six days old.
5. Experimental cheese	59.80	59.80	12.64	3.24	43.92	21.14	5.42	73.44	Seven weeks old.

From a study of these results it appears that ripening is accompanied by a process of respiration which results in the disappearance of oxygen and the production of an equivalent amount of carbon dioxide. In the early period of ripening the carbon dioxide from this source is augmented by the carbon dioxide produced by bacteria which decompose milk sugar. During this period the carbon dioxide is much higher and the nitrogen lower than would result from a simple process of respiration. The diffusion of gases tends to reduce this excess of carbon dioxide after the disappearance of milk sugar and the mixture of gases approaches the composition which would result from a process removing the oxygen from the air and producing an equivalent amount of carbon dioxide. The percentage of oxygen is always low and any aerobic organism which thrives within the cheese must be capable of obtaining its oxygen from a very dilute atmosphere of oxygen.

Various investigations have demonstrated that molds will not

grow in an atmosphere of carbon dioxide or of hydrogen. Similar results have been reported already in this investigation.<sup>3</sup> By these cultures it was further shown by the removal of the inhibiting gas that the spores present were not injured by either gas since all species developed quickly after the gases were removed. It is physically impossible for molds such as *Penicillium* to develop normally within a dense mass of substratum. Colonies develop only near and upon the surface of the culture medium although the vegetative hyphae of some species may extend into the mass a distance of 1 cm. or even farther. In cheese, however, hyphae can rarely be demonstrated 3 mm. below the surface. The presence of hyphae in deeper areas not connected with a surface colony raises a presumption of the presence of air spaces. Bitting has shown that *Penicillium expansum* will not grow in a vacuum, thus showing conclusively its dependence upon free oxygen. In seeking the explanation of the dominance of *Penicillium roqueforti* under the conditions shown to exist in Roquefort cheese it became desirable to study the effect of varying percentages of carbon dioxide upon a series of species. In the light of previous work, the carbon dioxide is regarded by the authors as an inert gas serving to dilute the atmospheric air. Increased percentages of this gas, therefore, would reduce the amount of available oxygen proportionally. A critical percentage is not to be expected but rather a gradual reduction of activity as the amount of oxygen present becomes reduced so that growth will be gradually decreased and ultimately stopped. For this purpose cultures of twenty-two species of *Penicillium* and five species of *Aspergillus* were made in Czapek's solution<sup>4</sup> agar slanted in test tubes in the ordinary way. During several years of work this medium has been found to give good average colonies of all the species used. A control set was carried under ordinary atmospheric conditions as a basis for comparison of results.

<sup>3</sup> Thom: U. S. Dept. of Agric., Bureau of Animal Industry, Bulletin 118, p. 90.

<sup>4</sup> Czapek's solution as given by Dox, A. W., U. S. Dept. of Agric., Bureau of Animal Industry, Bulletin 120: 1000 cc. of water, 0.5 gram magnesium sulphate, 1 gram dipotassium phosphate, 0.5 gram potassium chloride, 2 grams sodium nitrate, 0.01 gram ferrous sulphate, 30 grams cane sugar and 15 grams agar. The author has substituted dipotassium phosphate for monopotassium phosphate as used by Dox because he prefers a neutral solution.

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*First experiment.* Three sets of cultures were prepared. One was kept in a crate upon the table as a control. The other sets were put into two Novy culture jars and sealed up for nineteen days. All three groups were kept close together upon the laboratory table in diffused light, and at ordinary room temperatures. When examined the control series showed good normal colonies of the type already familiar from repeated study. Samples of the air in the Novy jars were removed and analyzed for carbon dioxide. In jar No. 1, this analysis showed 24.8 per cent, and in No. 2, 24.9 per cent of carbon dioxide. The cultures were then removed and compared with the control cultures. Following a practice previously used by one of us<sup>5</sup> a fully normal colony is designated in the tabulation of results as 1.0, and the observer's judgment as to amounts less than normal is expressed in tenths. To one who has cultivated these species many times the figures given in the table suggest the following interpretation. Species capable of developing normally within a few days show fully developed colonies, with perhaps one exception. *P. divaricatum* seems to be affected very quickly by the carbon dioxide since it shows about the amount of growth ordinarily produced in four days. Species requiring a week or more for normal growth ordinarily are retarded and in some cases prevented from reaching fully normal appearance. The species of *Aspergillus* except *A. fumigatus* were strongly reduced. In general the cultures had a retarded appearance resembling the appearance of colonies grown under cold conditions. Colonies nineteen days old had the appearance common to colonies seven to eight days old. The activities of the organisms seemed to be arrested as shown by the fresh bright colors and the lack of the shades of color both in spores and in mycelium usually seen in old cultures.

*Second experiment.* Since the jars used in the first experiment showed approximately 25 per cent of carbon dioxide at the close of the experiment, that figure was taken as a starting point for the second set of cultures. Two jars were prepared as before to contain the same set of species and were permitted to grow for eight days. The gases present were then analyzed.

	CO <sub>2</sub> in beginning	CO <sub>2</sub> at end
Jar 1.....	24.8	37.7
Jar 2.....	23.6	37.8

<sup>5</sup> Thom: *loc. cit.*



Examination of the cultures showed a reduction of vigor for most species. However, a number of forms as reported in the table show nearly normal colonies at the end of the experiment. Certain species are greatly reduced, but the number of strong colonies includes a considerable variety of species.

*Third experiment.* The Novy jars were prepared again with approximately 50 per cent of carbon dioxide. The cultures stood seven days and samples of the air were then analyzed.

	CO <sub>2</sub> at beginning	CO <sub>2</sub> at end
Jar 1.....	49.0	52.0
Jar 2.....	48.0	46.0

Although there must have been a considerable production of carbon dioxide the percentage of this gas at the end of the experiment was very nearly the same as at the beginning. This is due to the absorption of carbon dioxide by the culture media.

The difference in the figures for carbon dioxide had no appreciable effect upon the cultures as recorded separately. The figures given in the table are the average. In this list, *P. roqueforti* and *P. expansum* give the best growth, but four colonies (0.5 or over) of *P. duclauxi*, *P. camemberti*, *P. biforme*, and *P. rugulosum* were produced. Even with *P. roqueforti* the colony failed to show the usual abundance of green conidia. It is evident that 50 per cent carbon dioxide is sufficient to eliminate some species and reduce the others but the ubiquitous and omnivorous *P. expansum* grew nearly as well as *P. roqueforti* under these conditions.

*Fourth experiment.* The series was prepared a fourth time with carbon dioxide as 75 per cent of the mixture of gases used. The jars stood eight days. When examined carbon dioxide was 69.2 per cent of the mixture in the one jar successfully sampled. Roquefort mold alone of the species showed fair colonies (0.5 or over). *P. duclauxi* showed some colored fruit and the others germination with more or less submerged mycelium only.

The detailed results of all four experiments are given in table II.

Comparison of these cultural results with the data from gas analysis indicates that *P. roqueforti* alone is capable of growing to any marked extent under the conditions regularly found in Roquefort cheese. The activity of this species even is greatly reduced by the scarcity of oxygen. Inspection of the gas analysis table shows that the cheese containing 7 per cent of free oxygen had produced far more mold than the others. Certain analyses

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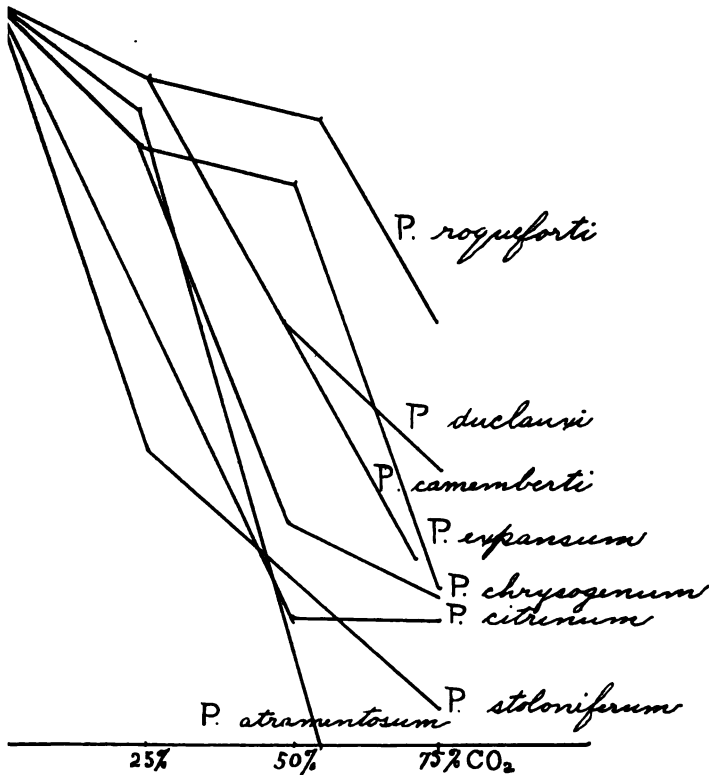
TABLE II.

*Cultures of Penicillium and Aspergillus grown in Novy jar with mixture of air and carbon dioxide.*

(Normal colonies designated 1.0, successive reductions in growth represented in tenths except that germination of spores alone is designated 0.1 and any further growth by increased figures.)

	CONTROL	CO <sub>2</sub> 0 AT START 25 PER CENT AT END	CO <sub>2</sub> 24.8 PER CENT AT START 37.7 PER CENT AT END	CO <sub>2</sub> 49 PER CENT AT START 53 PER CENT AT END	CO <sub>2</sub> 75 PER CENT AT START 90.2 PER CENT AT END
<i>P. camemberti</i> .....	1.0	1.0	0.9	0.5-6	0.2
<i>P. claviforme</i> .....	1.0	0.8	0.5	0.2	0.1
<i>P. granulatum</i> .....	1.0		0.85	0.35	0.2
<i>P. italicum</i> .....	1.0	0.8	0.9	0.45	0.2
<i>P. expansum</i> .....	1.0	0.9	0.8	0.75	0.2
<i>P. citrinum</i> .....	1.0	0.8	0.6	0.15	0.1
<i>P. purpurogenum</i> .....	1.0		0.4	0.2	0.2
<i>P. roqueforti</i> .....	1.0	1.0	0.9	0.85	0.5
<i>P. duclauxi</i> .....	1.0		0.9	0.55	0.3
<i>P. 24</i> .....	1.0	1.0	0.8	0.35	0.1
<i>P. chrysogenum</i> .....	1.0	1.0	0.9	0.3	0.2
<i>P. stoloniferum</i> .....	1.0	1.0	0.4	0.2	0.1
<i>P. divaricatum</i> .....	1.0	0.6	0.4	0.35	0.1
<i>P. atramentosum</i> .....	1.0	1.0	0.85	0.1	0.0
<i>P. biforme</i> .....	1.0		0.8	0.55	0.0
<i>P. 43</i> .....	1.0	0.8	0.3	0.15	0.0
<i>P. spinulosum</i> .....	1.0	1.0	0.6	0.35	0.0
<i>P. rugulosum</i> .....	1.0	1.0	0.7	0.6	0.0
<i>P. glaber</i> (Cit.).....	1.0	1.0	0.7	0.25	0.0
<i>P. 2479</i> .....	1.0	1.0	0.6	0.45	0.0
<i>P. 2481</i> .....	1.0	0.3	0.4	0.15	0.0
<i>A. flavus</i> .....	1.0	0.5	0.7	0.35	0.0
<i>A. niger</i> .....	1.0	0.6	0.6	0.15	0.0
<i>A. ochraceus</i> .....	1.0	0.3	0.3	0.15	0.0
<i>A. fumigatus</i> .....	1.0	1.0	0.8	0.25	0.0
<i>A. flavus. var.</i> .....	1.0	0.6	0.7	0.4	0.2

not included suggest that the percentage of oxygen frequently falls even lower, especially in freshly made cheeses. If such cheeses are paraffined or closely covered with tin-foil no increase in oxygen is probable. In experimental work many cheeses have been produced which were open enough within to favor mold growth but



ves showing relative activity of species beginning at 1.0 for normal control culture and showing the relative reduction of activity with res containing 25, 50 and 75 per cent carbon dioxide.

ich no growth developed in many months. Abundant mold s were present. These experiments indicate that the failure en was due to the scarcity of oxygen within these cheeses. ss in ripening cheeses of the Roquefort group will depend controlling the gas content of the cheeses within the range minance of the typical Roquefort species of *Penicillium*. r these conditions the growth of *P. roqueforti* while slowly exceeds that of any other species studied. In actual prac- cultures made from many specimens of Roquefort of different ls, also from Gorgonzola and Stilton, have shown frequently cultures of this species while the few species found as con- nations have formed a very small part of the flora.

## 258 Dominance of Roquefort Mold in Cheese

### SUMMARY.

Analysis of the gas collected from the air spaces in Roquefort cheese (both imported and made experimentally) shows that the percentage of free oxygen is low, in no case among those tabulated rising above 7 per cent.

Carbon dioxide appears in proportions ranging from 21 to 40 per cent. The percentage of carbon dioxide is highest in comparatively fresh-made cheese where it arises both from respiration of the microorganisms present and as a by-product of the decomposition of lactose by bacteria. From the maximum figure the percentage falls slowly by diffusion.

No hydrocarbons were found. Hydrogen if present was in very small amount.

Cultures of twenty-two species of *Penicillium* and five species of *Aspergillus* grown in a Novy culture jar for nineteen days produced an atmosphere containing approximately 25 per cent of carbon dioxide. Cultures of the same species in the same jar with an initial mixture of air and 25 per cent of carbon dioxide showed 37 per cent of carbon dioxide at the end of eight days with marked reduction in the activity of certain species.

Cultures of the same series of fungi in jars with approximately 50 per cent of carbon dioxide in the initial mixture left the mixture approximately unchanged at the end of seven days. Fungous growth was stopped for certain species, greatly reduced for all, but six of the species tested produced very considerable growth.

Cultures of this series of species grown for eight days in jars with 75 per cent of carbon dioxide in the initial mixture showed that *Penicillium roqueforti* alone was able to produce fairly strong colonies in this mixture.

A mixture of 75 per cent of carbon dioxide with air gives approximately 5 per cent of free oxygen. The close correspondence between the results of gas analysis and comparative culture, indicates that the low percentage of oxygen in the open spaces within the cheese accounts for the dominant activity of *Penicillium roqueforti* in Roquefort and related types of cheese.

## NOTE ON THE VOLATILITY OF SULPHURIC ACID WHEN USED IN VACUUM DRYING.

By H. C. GORE.

(From the Bureau of Chemistry of the U. S. Department of Agriculture, Washington, D. C.)

(Received for publication, June 11, 1913.)

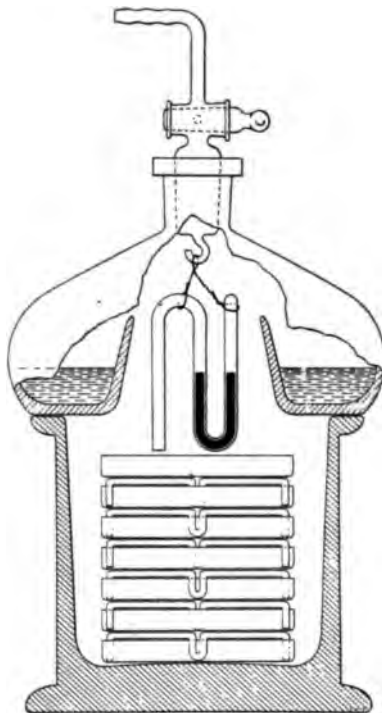
Highly perfected methods for drying perishable tissues and foods have been devised by Benedict and his coworkers,<sup>1</sup> and by Trowbridge,<sup>2</sup> and it is anticipated that these methods will soon come into general use, as pumps of high capacity giving high vacua are now generally available. In all of these methods sulphuric acid is used as the desiccant. Though possessing a marked advantage by virtue of its property of absorbing vapors of alcohol, ether and other substances as well as water vapor, it possesses the disadvantage of becoming coated over with a surface layer of dilute acid so that it is necessary to agitate the container gently at intervals to promote rapid drying. In addition, its corrosive nature marks it as a reagent the use of which is to be avoided wherever possible. In the course of experiments in drying in high vacuum over sulphuric acid the writer obtained indications that sulphuric acid is sensibly volatile under these conditions; for example, it was observed that flour so dried darkened perceptibly. No idea was had however of the extent to which the acid is volatile. Accordingly the experiment described below was made. The data obtained give an idea of the rate at which sulphuric acid will distil in vacuum of the order of 1 mm.

The experiment consisted in exposing powdered potassium hydroxide in a Hempel desiccator containing sulphuric acid for a long time at room temperatures at a vacuum of somewhat less than 1 mm. The details are as follows:

<sup>1</sup> Benedict and Manning: *Amer. Chem. Journ.*, xxvii, p. 340, 1902; *Amer. Journ. of Physiol.*, xiii, p. 309, 1905.

<sup>2</sup> U. S. Department of Agriculture, Bureau of Chemistry, Bulletin 122, p. 219.

A Hempel desiccator well cleaned and dried was charged with 200 cc. of 95 per cent C. P. sulphuric acid taken from a freshly opened bottle. A mercury manometer was suspended from the hook attached to the stop-cock in the cover by means of a platinum wire. In the lower part of the desiccator was placed a series of Petri dishes resting one upon the other, separated from each



ARRANGEMENT OF PETRI DISHES, MANOMETER, AND SULPHURIC ACID IN HEMPEL DESICCATOR.

other by glass triangles. The corners of the triangles were bent down to prevent the dishes from touching the sides of the desiccator. The arrangement is shown in the accompanying figure. Just before being placed in position each dish was charged with 5 to 10 grams of potassium hydroxide spread out in a thin layer. The ground edge of the desiccator and the stop-cock were lubricated with a mixture of vaseline and gum chicle. With the excep-

tion of a small portion of this lubricant which collected about the inner edge of the ground surface, no organic matter was present in the desiccator. The powdered potassium hydroxide used was so free from sulphates that on acidifying a test portion, adding solution of barium chloride and digesting on the steam bath, a faint, almost invisible precipitate collected in the center of the beaker on stirring.

A May-Nelson pump was used in exhausting. The vacuum obtained was slightly less than 1 mm. and remained unchanged from May 21, 1912, to January 13, 1913, 237 days. The desiccator was then opened and sulphate determined in the potassium hydroxide in each dish.

The results are given in the following table.

NUMBER OF DISH*	1	2	3	4	5	6	7	TOTAL
Sulphuric acid distilled, gm. ....	0.2133	0.0440	0.0169	0.0061	0.0072	0.0026	0.0029	0.2930

\* Numbered from top to bottom.

The amount of barium sulphate obtained in a blank on the reagents was not weighable. In 237 days therefore 0.2930 gram of sulphuric acid distilled from the acid in the cover of the desiccator and collected in the potassium hydroxide. The area of sulphuric acid exposed was about 188.5 sq. cm. The total loss was therefore 1.236 mgm. per day or 0.00656 mgm. per sq. cm. per day.

Experiments at the Bureau of Chemistry in drying samples of fruits in high vacuum show that lime may be successfully substituted for sulphuric acid.





## THE RACEMIZATION OF PROTEINS AND THEIR DERIVATIVES RESULTING FROM TAUTO- MERIC CHANGE. PART II.

### THE RACEMIZATION OF CASEIN.

By H. D. DAKIN AND H. W. DUDLEY.

(From the Herter Laboratory, New York.)

(Received for publication, June 10, 1913.)

In a recent communication<sup>1</sup> one of us has reported experiments on the racemization of gelatin by dilute alkali at low temperatures and shown that the process is well explained by assuming a keto-enol tautomerism of the  $-\text{CH}-\text{CO}-$  groups in the protein complex, and further that the optical activity or inactivity of the amino-acids obtained by hydrolysis of this racemized protein may be indicative of the positions of the respective amino-acids in the molecule.

In the hope of gaining further insight into the structure of proteins this work is being extended to other proteins and peptides and the object of this paper is to describe experiments made on the action of dilute alkali on casein.

A 10 per cent solution of casein in  $\frac{N}{2}$  sodium hydroxide was digested at 37°C. until the rotation fell to a constant value. On acidifying this solution "racemized casein" was precipitated and finally obtained as a powder closely resembling ordinary casein. In analysis it was found to have C = 53.5, N = 12.5, H = 7.03 per cent, while a typical analysis of ordinary casein is C = 52.9, N = 15.6, H = 7.05 per cent. Qualitative tests of the racemized product revealed a very slight phosphorus content but a fair amount of sulphur.

Simultaneously with racemization of the protein, hydrolysis also took place, so that on saturating the solution from which "racemized" casein had been removed with ammonium sulphate an

<sup>1</sup> This Journal, xiii, p. 357, 1912.

albumose-like body, "racemized" caseose, was precipitated. The filtrate from this substance gave a precipitate with Hedin's tannic acid mixture,<sup>2</sup> as also the biuret test and tyrosine reaction with Millon's reagent. It therefore contained further simpler hydrolytic products.

The isolation of "racemized" casein and caseose from the digestion mixture is a point of some value. For in the work on gelatin no products were isolated but the mixtures were hydrolyzed as they stood. Thus, as was previously pointed out, the optical activity of certain of the amino-acids might be due simply to the fact that they were rapidly split off from the protein molecule by hydrolysis and so escaped racemization. This objection cannot be raised in connection with the amino-acids derived from "racemized" casein and caseose, and the occurrence of optically active amino-acids must be taken as evidence in favor of the view that they occupy terminal positions in the peptide chains.

The optical properties of corresponding amino-acids obtained by the hydrolysis of "racemized" casein and caseose were in every instance identical. Alanine was present in quantities so small that repeated recrystallization failed to secure a fraction of the pure substance. But a valine-alanine mixture rich in the latter was obtained, and this proved to be inactive in both aqueous and hydrochloric acid solutions. Thus inactive alanine was undoubtedly present. Whether any active alanine occurred could not be decided. Both *dextro* and inactive valine were isolated without much difficulty, and large quantities of *laevo* and inactive leucine were found. Phenylalanine, tyrosine, arginine, lysine, histidine, aspartic and glutamic acids were all inactive, while proline was active. An observation of some interest may be made here. The question as to the pre-existence of proline as such in the protein molecule has been raised by E. Fischer and often discussed. His experiments<sup>3</sup> and those of Kossel<sup>4</sup> on this subject have made it most probable that proline is a primary and not a secondary product. The fact that glutamic acid and arginine (ornithine) from "racemized" casein are optically inactive, while the proline is active, precludes the possibility of the latter being derived from

<sup>2</sup> *Journ. of Physiol.*, xxxii, p. 468.

<sup>3</sup> *Zeitschr. f. physiol. Chem.*, xxxv, p. 227, 1902; *ibid.*, lxx, p. 118, 1910.

<sup>4</sup> *Ibid.*, xl, p. 311, 1903.

the former during the process of hydrolysis, and is direct evidence in favor of the view that proline itself is a protein constituent. The activity of proline obtained from "racemized" gelatin, casein and caseose would suggest that the carboxyl group may be free. In this case the peptide linkage must occur with the nitrogen atom of the proline molecule, a conclusion which has been reached by Kossel and Gawrilow<sup>5</sup> from investigations concerning the free amino-groups in proteins.

Of course the possibility of linkage by means of the carboxyl group is not absolutely precluded by our results, since in the communication on gelatin it was pointed out that prolylglycine is apparently not racemized by treatment with dilute alkali. Experiments with peptides of known structure are being carried out to elucidate this point.

The total inactivity of the bases, histidine, arginine and lysine may be regarded as evidence that they were attached to the protein molecule by condensation of their carboxyl groups with the amino groups of other constituents, results which indicate a similar linking to that of these bases in certain protamines investigated by Kossel and his collaborators.<sup>6</sup>

Both "racemized" casein and caseose gave the Adamkiewicz-Hopkins reaction for tryptophane, but attempts to isolate it failed, owing to the surprising fact that both substances were entirely resistant to the action of trypsin, and also of pepsin and erepsin.<sup>7</sup>

This absolute stability against the common proteolytic enzymes suggests that the racemization due to the tautomeric change  $>\text{CH}-\text{CO}-\text{NH}- \rightleftharpoons >\text{C}=\text{C.OH}-\text{NH}-$  must be complete for every such group in the protein molecule. Otherwise, if a few groups ( $>\text{CH}-\text{CO}-\text{NH}-$ ) retaining their natural configuration remained intact, one might expect enzymes to effect hydrolysis at such points yielding simpler peptones and peptides. So far no evidence of such partial splitting has been obtained.

The racemization of proteins at low temperatures by the action of dilute alkalis may in part account for the wide variations which

<sup>5</sup> *Zeitschr. f. physiol. Chem.*, lxxxi, p. 274, 1912.

<sup>6</sup> *Zeitschr. f. physiol. Chem.*, lxxii, p. 486, 1911; lxxviii, p. 402, 1912; lxxxiv, p. 1, 1913.

<sup>7</sup> *This Journal*, xv, p. 271, 1913.

have been observed in the optical properties of tryptophane,<sup>8</sup> and for the isolation of racemic tryptophane by Ellers.<sup>9</sup>

In conclusion, these results raise a doubt as to whether it will ever be possible by existing methods to synthesize a naturally occurring protein. For treatment of compounds containing peptide linkages with alkali occurs in all these processes, and with large molecules of this character racemization of the type described in this paper would most probably occur.

In view of these studies it would appear to be of importance to avoid the use of alkali in the extraction and purification of proteins. This procedure is often employed but must effect partial racemization and hence is objectionable. The difficulty of carrying out tryptic digestion until the biuret test becomes absolutely negative is a matter of common experience. This is in all probability due to partial racemization of the protein, either occasioned by its mode of preparation or actually occurring during digestion. When complete tryptic digestion is wanted it is obviously necessary to keep the alkalinity of the solution as low as possible.

A comparative table showing the optical properties of the amino-acids derived from "racemized" gelatin, casein and caseose is here appended.

	GELATIN	CASEIN	CASEOSE
Alanine.....	<i>d</i> and inactive	inactive (and <i>d</i> ?)	inactive (and <i>d</i> ?)
Valine.....		<i>d</i> and inactive	<i>d</i> and inactive
Leucine.....	inactive	<i>l</i> and inactive	<i>l</i> and inactive
Tyrosine.....		inactive	inactive
Phenylalanine...	inactive	inactive	inactive
Proline.....	<i>l</i>	<i>l</i>	<i>l</i>
Aspartic acid....	inactive	inactive	inactive
Glutamic acid....	<i>d</i>	inactive	inactive
Arginine.....	inactive	inactive	inactive
Lysine.....	<i>d</i>	inactive	inactive
Histidine.....	inactive	inactive	inactive

These investigations are being continued.

<sup>8</sup> Cf. *Zeitschr. f. physiol. Chem.*, lv, pp. 74 and 412, 1908.

<sup>9</sup> *Biochem. Zeitschr.*, vi, p. 272, 1907.

## EXPERIMENTAL.

*Preparation of "racemized" casein and caseose.*

500 grams of commercial casein are shaken up with 5 liters of  $\frac{N}{2}$  sodium hydroxide until a homogenous mixture is obtained. After standing for a day in the incubator at 37°C. the liquid is filtered from the flocculent precipitate formed and the clear brown filtrate is returned to the incubator for 18–20 days when the rotation is found to have dropped to a constant value. The change in rotation of such a solution is from about  $-5^\circ$  to  $-3^\circ$ . A considerable amount of ammonia is evolved during incubation.

The brown liquid is then neutralized, while still warm, with sulphuric acid and becomes slightly turbid. Glacial acetic acid is added in small quantities at a time with constant stirring until no more precipitation of "racemized" casein occurs. The casein comes out in white flakes which adhere to the stirring rod, forming a plastic, dough-like mass which is best removed from the solution in this way. The small amount remaining in the liquid settles and adheres to the bottom of the vessel when the liquid may be poured off and the remainder of the "racemized" casein scraped out. The plastic substance is then pulled into small pieces which are dropped into water, which is subsequently poured off. The process is repeated until the wash water no longer shows an acid reaction to litmus. The racemized casein is then placed on porous plate and allowed to dry at room temperature for several days. It finally assumes a brown appearance and becomes friable. It may then be reduced to a fine white powder, resembling ordinary casein in appearance.

The average yield from 500 grams of casein is 100 grams of the "racemized" product.

*Analysis of "racemized" casein.*

The substance was dried in a steam oven.

0.1548 gram gave 0.3040 gram  $\text{CO}_2$  and 0.0979 gram  $\text{H}_2\text{O}$ .

0.4940 gram required by Kjeldahl's method 16.75 cc.  $\frac{N}{4}$  HCl whence

C = 53.55 per cent, H = 7.03 per cent, N = 12.5 per cent.

The acid liquid after removal of "racemized" casein is concentrated on the water bath and saturated with ammonium sulphate. A dark brown, sticky mass of "racemized" caseose is

precipitated, which, after purification from adhering salts and acetic acid, if spread in thin layers and exposed to the air for some time, becomes hard and brittle and may be ground in a mortar to a fine white powder. This substance is soluble to a practically unlimited extent in water, and exhibits more acidic properties than "racemized" casein.

A determination of the amount of complete hydrolysis brought about by the alkali was made by estimating the total nitrogen of the digestion liquid and comparing it with the nitrogen non-precipitable by phosphotungstic acid. A tenth of the total nitrogen was thus found to remain in solution.

#### *Hydrolysis of "racemized" casein and caseose.*

340 grams of dry "racemized" casein and 600 grams of moist, crude "racemized" caseose were hydrolyzed with hydrochloric acid for investigation of the amino-acids according to Fischer's ester method.

*Alanine* was obtained in a fraction together with valine which was inactive in both cases. Other mixtures whose analyses indicated the presence of some alanine were active, but this may have been due to the presence of *d*-valine.

*Valine* was obtained inactive in mixture with alanine, and also in mixtures of *r*- and *d*-valine containing over 50 per cent of the latter variety.

*Leucine* was obtained pure in the inactive form, and also in mixtures of *l*- and *r*-leucine. Mixtures of leucine and valine which showed optical activity were also obtained.

*Tyrosine* was isolated by hydrolyzing the digestion mixture of 50 grams of casein as it stood with hydrochloric acid. The solution was evaporated *in vacuo*, the residue, dissolved in water, treated with animal charcoal and filtered. Ammonia was added, the solution concentrated and tyrosine allowed to crystallize out. The crude product was washed with hot glacial acetic acid, dissolved in hydrochloric acid and liberated by adding ammonia. It crystallized in characteristic form. It was found to be inactive in hydrochloric acid solution, a 25 per cent solution being examined in a 2.2 dm. tube. It follows that the tyrosine in both "racemized" casein and caseose is inactive.

*Phenylalanine* was obtained pure in large quantities and proved to be inactive in hydrochloric acid solution in both cases.

*Proline*. The rotation of the proline isolated from "racemized" casein indicated the presence of 48.9 per cent of the *laevo* form, while from "racemized" caseose a specimen was obtained which contained 30.7 per cent of the active variety. Since proline suffers partial racemization during the process of isolation it is probable that no racemization occurred before hydrolysis.

*Aspartic and glutamic acids* were obtained from both substances. The yields of the former were small, but both acids were completely inactive.

*Arginine, lysine and histidine* were isolated by the method of Kossel and Kutscher from 100 grams dry "racemized" casein and 125 grams crude "racemized" caseose respectively. The histidine, arginine, and lysine fractions were all optically inactive. The lysine fractions proving to be totally inactive rendered it unnecessary to isolate ornithine from them.





## THE ACTION OF ENZYMES ON RACEMIZED PROTEINS, AND THEIR FATE IN THE ANIMAL BODY.

By H. D. DAKIN AND H. W. DUDLEY.

(From the Herter Laboratory, New York City.)

(Received for publication, June 12, 1913.)

In this communication we are concerned with the behavior of "racemized" casein and caseose described in the preceding paper when subjected to the actions of pepsin, trypsin and erepsin. It was surprising to find that these simple derivatives of casein were both entirely resistant to the above enzymes, no hydrolysis taking place in any case with experiments *in vitro*. It was clearly of interest to determine what would be the fate of these substances in the animal body, since the results of such experiments might throw light upon the mechanism of intestinal absorption. On feeding to a dog by mouth, it was found that both substances were excreted unchanged in the feces, no absorption at all having taken place in the intestine.

It thus appears probable that the intestine is incapable of absorbing, as such, even substances of the complexity of albumoses, and can only take up bodies of simpler molecular structure.

It might be objected against this view that absorption might not take place with a body whose behavior towards enzymes is so unusual, while a substance of similar complexity, but normal (*i.e.*, digestible) to enzymes conceivably could be absorbed. But Dakin<sup>1</sup> has shown that certain simple optical enantiomorphs are equally well absorbed although enzymes may attack one stereoisomer much more readily than the other, so that the above argument seems improbable. It is more likely that intestinal absorption is a physical process, and it would be of great interest to ascertain the highest degree of molecular complexity that constituents of proteins and other bodies may possess and at the same time undergo absorption in the intestine. We shall attempt to obtain

<sup>1</sup> This *Journal*, iv, p. 437, 1908.

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simpler fragments of the "racemized" protein molecule in the hope of further elucidating this question, *i.e.*, to what extent the protein molecule must be broken down before the fragments become capable of absorption in the intestine.

Given subcutaneously in concentrated aqueous solution to a dog, "racemized" caseose was excreted unchanged in the urine, the animal showing no symptoms.

The action of putrefactive bacteria was also tried on the two substances. The "racemized" casein remained unchanged, but the caseose was slowly attacked yielding indol and other products.

### EXPERIMENTAL.

*Methods.* Solutions of the two substances were made under conditions favoring the action of the particular enzyme under investigation. Similar solutions of ordinary casein were prepared and used as controls with pepsin and trypsin, and a solution of Witte's peptone was employed in the case of erepsin.

Equal amounts of the enzyme solution were then added to the three solutions, from which 5 cc. were immediately pipetted off and the total nitrogen estimated according to Kjeldahl. Estimations of the non-precipitable nitrogen were also immediately made by taking a known amount of the solutions, precipitating with a known volume of a suitable reagent, either Hedin's "tannic acid mixture"<sup>2</sup> or a 33 per cent solution of trichloroacetic acid, and then estimating the nitrogen in the filtrate by Kjeldahl's method, after the mixtures had stood for eighteen hours.

Periodically such non-precipitable nitrogen determinations were carried out, and thus it was possible to follow the course of the hydrolyses in the solutions, which were kept at a temperature of 37°C. in an incubator.

It will be noted that in the case of "racemized" caseose the non-precipitable nitrogen was comparatively high from the beginning of the experiment. This was largely caused by the presence of some ammonium sulphate derived from the salting-out process.

<sup>2</sup> *Journ. of Physiol.*, xxxii, p. 468.

*Comparative hydrolyses of ordinary casein, "racemized" casein, and caseose by pepsin.*

Four grams of each of the substances were dissolved in 100 cc. of  $\frac{N}{10}$  HCl. To each of these solutions 0.1 gram of Merck's pepsin, dissolved in 5 cc. of water, was added together with a few drops of toluene. Total and non-precipitable nitrogen determinations were made immediately. Precipitation was effected with the ordinary casein and "racemized" casein by adding 5 cc. of a 33 per cent trichloroacetic acid solution to 10 cc. of the solutions. With the solution of "racemized" caseose this reagent gave only a turbid, milky emulsion, so that in this case 10 cc. of the solution were precipitated with 10 cc. of tannic acid mixture. After standing for eighteen hours the non-precipitable nitrogen was determined in the filtrate. The solutions were kept at 37°C. and periodically non-precipitable nitrogen determinations were made. The results are given in the following table.

SUBSTANCE	TOTAL NITROGEN PER 100 cc.	NON-PRECIPITABLE NITROGEN PER 100 cc.		
		0 hours	24 hours	48 hours
	gram	gram	gram	gram
Casein.....	0.434	0.042	0.245	0.294
"Racemized" casein.....	0.406	0.042	0.063	0.063
"Racemized" caseose.....	0.497	0.259	0.259	0.259

*Comparative hydrolyses of ordinary casein, "racemized" casein and caseose by trypsin.*

Ten grams of ordinary casein and 10 grams of "racemized" casein were dissolved in 200 cc. of 0.8 per cent  $\text{Na}_2\text{CO}_3$  solution. Ten grams of "racemized" caseose were dissolved in 200 cc. of water containing 1.6 grams of  $\text{Na}_2\text{CO}_3$ . To each of these solutions were added 5 cc. of a trypsin solution prepared from ox pancreas by Hedin's method<sup>3</sup> and 2 cc. of toluene. Total nitrogen and non-precipitable nitrogen determinations were made as in the above experiment, except that equal volumes of the solutions

<sup>3</sup> *Journ. of Physiol.*, xxxii, p. 468.

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and of tannic acid mixture (10 cc.) were used for the estimation of non-precipitable nitrogen in all cases. The digestions were carried out at 37°C. The results are as follows:

SUBSTANCE	TOTAL NITROGEN PER 100 cc.	NON-PRECIPITABLE NITROGEN PER 100 CC.			
		0 hours	24 hours	48 hours	72 hours
	gram	gram	gram	gram	gram
Casein.....	0.595	0.025	0.329	0.399	0.441
"Racemized" casein...	0.560	0.063	0.069	0.070	0.070
"Racemized" caseose..	0.504	0.217	0.245	0.245	0.245

As a confirmatory experiment 100 cc. of each of the above solutions of "racemized" casein and caseose were taken and 25 cc. of the trypsin solution were added. The nitrogen determinations were as follows:

SUBSTANCE	TOTAL NITROGEN PER 100 cc.	NON-PRECIPITABLE NITROGEN PER 100 CC.	
		0 hours	24 hours
	gram	gram	gram
"Racemized" casein.....	0.511	0.154	0.168
"Racemized" caseose.....	0.490	0.273	0.287

The slight rise in non-precipitable nitrogen was undoubtedly due to autolysis of the added enzyme solution.

### *Comparative hydrolyses of Witte's peptone, "racemized" casein and caseose by erepsin.*

A solution of erepsin was prepared by digesting the mucosa of a dog's intestine at room temperature for fifteen hours with physiological saline solution, filtering through muslin, and employing the extract so obtained without further purification. A determination of the amount of autolysis in this solution showed that the non-precipitable nitrogen rose from 0.119 to 0.290 gram per 100 cc. liquid in forty-eight hours at 37°C. Five grams of Witte's peptone were dissolved in 100 cc. of water. The solution was faintly alkaline to litmus. 2.5 grams of racemized casein were dissolved to a slightly turbid solution in 100 cc. of 0.2 per cent  $\text{Na}_2\text{CO}_3$  solution; the reaction to litmus was neutral. Five grams

of "racemized" caseose were dissolved up in 100 cc. of water. This solution was acid to litmus. Sodium carbonate solution was added until the alkalinity of the liquid was of the same order as that of the peptone solution.

To each of these solutions were added 10 cc. of the erepsin extract and 1 cc. of toluene. The usual nitrogen determinations were made, tannic acid mixture being used as precipitant. The results were as follows:

SUBSTANCE	TOTAL NITROGEN PER 100 cc.	NON-PRECIPTABLE NITROGEN PER 100 cc.		
		0 hours	24 hours	48 hours
	<i>gram</i>	<i>gram</i>	<i>gram</i>	<i>gram</i>
Witte's peptone.....	0.714	0.189	0.567	0.672
"Racemized" casein.....	0.280	0.023	0.046	0.065
"Racemized" caseose.....	0.483	0.168	0.196	0.210

The slight rises in the non-precipitable nitrogen of the "racemized" casein and caseose solutions are accounted for by the autolysis of the added erepsin extract.

*Fate of "racemized" casein and caseose in the animal body.*

Preliminary experiments showed that on giving small quantities of the two substances mixed with food to a dog, tests for proteins in the urine were negative.

The dog, after fasting, was put on a fixed diet of 100 grams of boiled rice and 20 grams of bone ash. At suitable intervals "racemized" casein was fed to the animal and the nitrogen elimination followed in twenty-four-hour periods. A comparative feeding of ordinary casein was made at the end of the experiment. The following table gives the results and indicates no absorption of nitrogen from the "racemized" protein.

PERIOD	TOTAL URINARY NITROGEN	SUBSTANCE FED	NITROGEN OF SUBSTANCE FED
	<i>grams</i>		<i>grams</i>
I	1.64	—	—
II	1.34	20 grams "racemized" casein	2.6
III	1.75	20 grams "racemized" casein	2.6
IV	2.31	20 grams ordinary casein	3.0

## 276 Action of Enzymes on Racemized Proteins

The feces passed after feeding "racemized" casein were extracted with 3 per cent sodium carbonate solution. The filtrate gave a strong biuret reaction and on acidifying "racemized" casein was precipitated.

Similar experiments with "racemized" caseose showed that it too passes through the gut without absorption and is readily recovered from the feces.

About 5 cc. of a concentrated aqueous solution of "racemized" caseose were injected subcutaneously into a dog. The animal showed no symptoms. After five hours urine was passed which gave a strong biuret reaction and a white turbidity with trichloroacetic acid, proving the presence of caseose. This urine gave no precipitate on boiling, nor on acidifying with acetic acid. A sample of urine collected before the injection was perfectly normal, and such was again the case a day after.

### *Action of putrefactive bacteria on "racemized" casein and caseose.*

Two grams of "racemized" casein and of "racemized" caseose were dissolved in 20 cc. of 0.8 per cent sodium carbonate solution containing traces of calcium chloride, magnesium sulphate and sodium phosphate.

The solutions were inoculated with small amounts of putrid pancreas infusion. After ten days the "racemized" casein was unaffected while in the caseose solution the organisms had slowly grown, decomposing the caseose with formation of indol and other products of putrefaction.

## STUDIES IN BACTERIAL METABOLISM. XI.

### DETERMINATION OF "UREA NITROGEN" IN CULTURES OF CERTAIN BACTERIA.

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The qualitative and quantitative measure of nitrogen degradation is one of the fundamental steps in studying cellular metabolism, for nitrogen is one of the most important structural elements which enter into the composition of the cell. In man, nitrogenous waste leaves the body largely in the form of urea, urea comprising about 80 per cent of the total nitrogen excreted. Most of this urea is formed in a special organ, the liver, although it is almost certain that muscles and perhaps other tissues form a small amount besides. The question naturally presents itself—do other unicellular organisms form urea, or is the ultimate degradation of nitrogen in such organisms largely ammonia. If it could be shown, for example, that unicellular organisms such as bacteria form urea, the bearing of such observations upon multicellular organisms is obvious. With the bacteria, which appear to excrete the greater part of their waste nitrogen as ammonia, urea formation would in reality be a product of intermediary metabolism, inasmuch as it represents nitrogen in a more complex state of organization than ammonia. Many bacteria can actually utilize urea as a source of nitrogen.

In order to throw some light upon this possibility—that of urea formation—a considerable number of bacteria representing various degrees of proteolytic activity have been grown in sugar-free and dextrose-containing standard nutrient broth made from meat juice and peptone (Witte's). This broth has been examined after inoculation with these bacteria at stated intervals, using the new

## 278 "Urea Nitrogen" in Bacterial Cultures

Folin<sup>1</sup> urea method, for the presence of "urea nitrogen," comparing the results obtained with suitable controls. According to Folin the presence of dextrose interferes with this determination until definite dilutions of the sugar have been reached. Observations on the uninoculated plain and dextrose broth controls indicate that the decrease of "urea nitrogen" in sugar broth containing 1 per cent of dextrose is but about 0.70 mgm. per 100 cc. less than that of the corresponding sugar-free broth in our media. What effect the salts of the organic acids may have had upon the determinations is not definitely known, but it is safe to say that the results are comparable, because the content of these substances is the same in both kinds of media. The rather striking uniformity of the results obtained in dextrose broth makes them of comparative interest, if not of relative value.

The method, briefly, is as follows: (1) 7 grams of pure, dry, ammonia-free potassium acetate are placed in a large test tube (8 inches  $\times$  1 inch), 1 cc. of 50 per cent acetic acid added, and 1 cc. of culture. The tube is closed with a rubber stopper containing a tube about 25 cm. long and 1 cm. in diameter. The mixture is heated to 155°C. for 20 minutes in a sulphuric acid bath, the condenser and stopper then washed down with a small amount of water, the solution made alkaline with 1 cc. of strong caustic soda and the ammonia blown into 10 cc. of  $\frac{N}{50}$  HCl by an air current. At the same time the free ammonia is determined in another portion of this broth in the same manner in uninoculated controls. Determinations are made in duplicate: the greatest difference between these duplicate determinations was 0.1 cc.  $\frac{N}{50}$  alkali, or 2.8 milligrams per 100 cc. of culture.

It is a fact that the "urea nitrogen" both in uninoculated media and in cultures of bacteria is far less than in urine, hence the percentage of error in these determinations is far greater even allowing for the very dilute standard solution used— $\frac{N}{50}$  HCl and NaOH respectively—than in corresponding urine determinations. The maximum error of the method as applied to bacterial cultures between duplicate determinations is roughly 2 per cent for the "urea nitrogen" and 1 per cent for the free ammonia determinations, hence the maximum total error is about 3 per cent.

<sup>1</sup>Folin: this *Journal*, xi, pp. 507-522, 1912.



Considering the very small amounts of nitrogen involved, this error cannot be regarded as excessive: it is the best that can be accomplished with the methods available at the present time.

The results are expressed respectively as milligrams of "urea" and ammonia nitrogen per 100 cc. of broth: the difference between the "urea" nitrogen and the ammonia nitrogen, after subtracting the corresponding controls (uninoculated broth), indicates the gain or loss in "urea" nitrogen by the various bacteria studied.

The tables show that the "urea" nitrogen of the cultures increases proportionately to the ammonia nitrogen, and to about the same relative degree: generally speaking the initial proportional excess of "urea" nitrogen over ammonia nitrogen is neither increased nor decreased noticeably. This would appear to indicate that products of intermediary metabolism of the nitrogen-containing constituents of the broth which can be reduced to ammonia by heating to 155°C. with the reagents used are not present in noteworthy amounts. Ammonia formation, the final step in the degradation of protein and protein derivatives by the commonly met with bacteria, is the best available index of proteolysis by bacteria.

The organisms studied are fairly representative proteolytically, hence the results shown in the tables cover the possibilities of this determination for bacteria in a moderately complete manner. It cannot be stated definitely that the observed increase in "urea" nitrogen is referable to urea. The very small amount of reacting substances would make their isolation very difficult. It is very probable that uninoculated broth contains at least some urea, derived from the meat which is a basis for the media. Whether this urea originally present is decomposed by bacterial growth and is replaced by other nitrogenous products of proteolytic origin, or whether it persists as urea cannot be stated. Even in the urine, where urea is present in considerable amounts, it is by no means definitely settled that the determination of "urea" nitrogen represents urea alone.

ORGANISM	DAY OF OBSERVATION	PLAIN BROTH				DEXTROSE BROTH			
		"Urea" N	Increase in "Urea" N	NH <sub>3</sub> -N	Increase in NH <sub>3</sub> N	"Urea" N	Increase in "Urea" N	NH <sub>3</sub> -N	Increase in NH <sub>3</sub> N
Control		14.70		8.40		+6.30	13.30	8.40	+4.90
Grass bacillus	1	15.75	1.05	8.40	0.00	+1.05	13.30	8.40	0.00
	3	17.65	3.15	9.80	1.40	+1.75	14.00	7.00	-1.40
	6	17.50	2.80	11.20	2.80	0.00	14.70	9.80	1.40
	9	21.00	6.30	15.40	7.00	-0.70	18.90	9.10	0.70
B. coli I	1	18.20	3.50	10.50	2.10	+1.40	14.70	8.40	0.00
	3	22.40	7.70	16.10	7.70	0.00	14.00	8.75	0.35
	6	27.30	12.60	18.90	10.50	+2.10	16.80	8.75	0.35
	9	30.10	15.40	20.30	11.90	+3.50	16.80	8.75	0.35
B. proteus II	1	16.80	2.10	9.80	1.40	+0.70	15.40	8.40	0.00
	3	21.70	7.00	14.70	6.30	+0.70	15.40	9.10	0.70
	6	33.60	18.90	25.90	17.50	+1.40	17.50	9.10	0.70
	9	41.30	26.60	36.40	28.00	-1.40	18.20	9.10	0.70
Sp. Finkler and Prior	1	16.80	2.10	9.10	0.70	+1.40	14.00	8.40	0.00
	3	22.40	7.70	14.00	5.60	+2.10	15.40	8.40	0.00
	6	26.60	11.90	18.90	10.50	+1.40	15.40	9.10	0.70
	9	28.70	14.00	21.70	13.30	+0.70	16.80	9.10	0.70
B. cloacae	1	16.10	1.40	9.10	0.70	+0.70	16.10	7.70	-0.70
	3	18.20	3.50	13.30	4.90	-1.40	16.80	9.10	0.70
	6	24.50	9.80	16.80	8.40	+1.40	19.60	11.00	3.50
	9	26.60	11.00	19.60	10.10	+1.80	35.00	17.50	9.10

B. dysenteriae Flexner.	1	9	16.80	2.10	9.10	0.70	+1.40	17.50	4.20	8.75	0.35	+3.85
	3	16.80	2.10	9.10	0.70	0.70	+1.40	13.30	0.00	8.40	0.00	0.00
	6	16.80	2.10	9.10	0.70	0.70	+1.40	16.80	3.50	8.40	0.00	+3.50
	9	17.50	2.80	10.15	1.75	1.75	+1.05	16.80	3.50	9.10	-0.70	+2.80
	Control		14.00		7.35		+6.65	13.30		7.00		+6.30
Hog cholera I (avirulent)	1	14.70	0.70	7.75	0.35	0.35	+0.35	13.30	0.00	7.00	0.00	0.00
	3	17.50	3.50	11.55	4.20	4.20	-0.70	13.30	0.00	7.70	0.70	-0.70
	6	22.40	8.40	19.25	11.90	11.90	-3.50	14.00	0.70	7.70	0.70	0.00
	9	23.10	9.10	19.25	11.90	11.90	-2.80	14.00	0.70	7.70	0.70	0.00
	Hog cholera II (virulent)	1	14.70	0.70	7.70	0.35	0.35	+0.35	13.30	0.00	7.35	0.35
	3	16.80	2.80	10.50	3.15	3.15	-0.35	12.60	-0.70	7.70	0.70	-1.40
	6	18.20	4.20	11.20	3.85	3.85	+0.35	13.30	0.00	7.70	0.70	-0.70
	9	18.20	4.20	11.90	4.55	4.55	-0.35	14.00	0.70	7.70	0.70	0.00
B. typhosus	1	14.00	0.00	7.35	0.00	0.00	0.00	13.30	0.00	7.00	0.00	0.00
	3	14.00	0.00	9.80	2.45	2.45	-2.45	13.30	0.00	7.35	0.35	-0.35
	6	15.40	1.40	9.80	2.45	2.45	-2.45	13.30	0.00	7.70	0.70	-0.70
	9	18.20	4.20	10.85	3.50	3.50	+0.70	14.00	0.70	7.70	0.70	-0.00
	Morgan bacillus	1	21.00	7.00	14.70	7.35	7.35	-0.35	14.00	0.70	8.05	1.05
3		21.70	7.70	15.75	8.40	8.40	-0.70	14.00	0.70	8.75	1.75	-1.05
6		21.70	7.70	17.50	10.15	10.15	-2.45	14.00	0.70	8.75	1.75	-1.05
9		22.40	8.40	17.50	10.15	10.15	-1.75	14.00	0.70	9.10	2.10	-1.40

ORGANISM	DAY OF OBSERVATION	PLAIN BROTH				DEXTRIN BROTH			
		"Urea" N	Increase in "Urea" N	NH <sub>3</sub> -N	Increase in NH <sub>3</sub> -N	"Urea" N	Increase in "Urea" N	NH <sub>3</sub> -N	Increase in NH <sub>3</sub> -N
Control		14.00		7.35		6.65		7.00	
Paratyphoid I	9	16.80	2.80	9.80	2.45	+0.35	0.00	7.70	0.70
Paratyphoid II	9	16.80	2.80	10.15	2.80	0.00	0.00	7.70	0.70
Paratyphoid III	9	16.80	2.80	10.15	2.80	0.00	0.00	7.70	0.70
Paratyphoid IV	9	16.10	2.10	9.80	2.45	-0.35	0.00	7.70	0.70
Paratyphoid V	9	16.80	2.80	10.15	2.80	0.00	-0.70	8.05	1.05
Paratyphoid VI	9	16.80	2.80	10.15	2.80	0.00	-1.40	7.35	0.35
Paratyphoid alpha I	9	17.50	3.50	14.00	6.65	-3.15	0.00	7.70	0.70
Paratyphoid alpha II	9	16.10	2.10	9.80	2.45	-0.35	-0.70	7.70	0.70
Paratyphoid alpha III	9	16.80	2.80	13.30	5.95	-3.15	0.00	7.70	0.70
Morgan bacillus I	9	22.40	8.40	18.20	10.85	-2.45	0.00	8.05	1.05
Morgan bacillus II	9	21.00	7.00	17.50	10.15	-3.15	0.00	9.10	2.10
Morgan bacillus III	9	22.40	8.40	18.20	10.85	-2.45	0.00	9.10	2.10
Morgan bacillus IV	9	21.70	7.70	17.50	10.15	-2.45	0.00	8.05	1.05
Hog cholera III	9	16.80	2.80	10.15	2.80	0.00	-0.70	7.70	0.70
Hog cholera IV	9	21.00	7.00	15.40	8.15	-1.05	0.00	7.35	0.35
Fowl cholera I	9	18.90	4.90	13.30	5.95	-1.05	-0.70	7.70	0.70
B. acidi lactici II	9	17.50	3.50	12.60	5.25	-1.75	0.00	7.84	0.84
B. acidi lactici III	9	17.50	3.50	11.55	4.20	-0.70	0.00	7.84	0.84
Swine plague	9	17.50	3.50	9.45	2.10	+1.40	-0.70	7.70	0.70
Shiga bacillus	9	16.10	2.10	9.45	2.10	0.00	-0.70	7.70	0.70
Cholera IV	9	33.60	19.60	28.95	19.60	0.00	-0.70	7.70	0.70
Diphtheria VIII	9	15.40	1.40	9.45	2.10	-0.70	-0.70	7.00	0.00

## THE INFLUENCE OF STARVATION UPON THE CREATINE CONTENT OF MUSCLE.

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(Received for publication, June 19, 1913.)

Considerable interest has been attached to the metabolism of creatine since Benedict<sup>1</sup> in 1907 noted its appearance in the urine in a series of experiments upon starving men, and subsequently, in collaboration with Diefendorf,<sup>2</sup> confirmed this observation upon a starving woman. This excretion of creatine in starving men was likewise noted independently by Cathcart,<sup>3</sup> while the experiments of Dorner,<sup>4</sup> and Mendel and Rose<sup>5</sup> on rabbits, and the observations of Underhill and Kleiner,<sup>6</sup> Richards and Wallace,<sup>7</sup> and Howe and Hawk<sup>8</sup> upon starving dogs would indicate that the excretion of creatine is common to starving mammals. It should be noted, however, that McCollum and Steenbock<sup>9</sup> have shown that in the pig inanition does not readily cause an elimination of creatine, which is in striking contrast to the ease with which its excretion is produced in the rabbit.

That creatine may appear in the urine in a variety of pathological conditions, especially in those associated with a loss in weight and under-nutrition, was pointed out by Benedict and one

<sup>1</sup> Benedict, F. G.: Carnegie Inst., Washington, Publ. No. 77, p. 386, 1907.

<sup>2</sup> Benedict and Diefendorf: *Amer. Journ. of Physiol.*, xviii, p. 362, 1907.

<sup>3</sup> Cathcart: *Journ. of Physiol.*, xxxv, p. 500, 1907.

<sup>4</sup> Dorner: *Zeitschr. f. physiol. Chem.*, lii, p. 225, 1907.

<sup>5</sup> Mendel and Rose: *this Journal*, x, p. 213, 1911. A discussion of the literature is given in this paper.

<sup>6</sup> Underhill and Kleiner: *ibid.*, iv, p. 165, 1908.

<sup>7</sup> Richards and Wallace: *ibid.*, iv, p. 179, 1908.

<sup>8</sup> Howe and Hawk: *Journ. of Amer. Chem. Soc.*, xxxiii, p. 215, 1911; also Howe, Mattill and Hawk: *this Journal*, x, p. 417, 1911.

<sup>9</sup> McCollum and Steenbock: *ibid.*, xiii, p. 209, 1912.

of us,<sup>10</sup> and subsequently confirmed by many workers. The more important conditions in which an elimination of creatine has been detected are carcinoma of the liver,<sup>11</sup> exophthalmic goitre,<sup>12</sup> muscular dystrophy,<sup>13</sup> anterior poliomyelitis,<sup>14</sup> typhoid fever,<sup>15</sup> pneumonia,<sup>16</sup> diabetes mellitus,<sup>17</sup> pernicious vomiting of pregnancy,<sup>18</sup> and following pregnancy. The elimination of creatine during the post partum resolution of the uterus in women was first noted by Shaffer,<sup>19</sup> and subsequently observed in dogs by Murlin.<sup>20</sup> In a recent communication, Mellanby<sup>21</sup> puts an entirely new interpretation upon this excretion of creatine. He has observed that the elimination is coincident with the secretion of milk, the excretion being of the same intensity where the uterus was removed in a case of Caesarian section.

As might be inferred from the foregoing observations, creatine is not normally a constituent of the urine in the adult, although Rose<sup>22</sup> has recently shown that it is constantly present in the urine of children, an observation which has been confirmed by Folin and Denis.<sup>23</sup> Just why children eliminate creatine is difficult to explain. In a still more recent communication, McCollum and Steenbock<sup>24</sup> report the excretion of creatine in growing pigs when on a high protein intake from certain sources. Since an equally high protein intake from another source may be without this

<sup>10</sup> Benedict and Myers: *Amer. Journ. of Physiol.*, xviii, p. 406, 1907.

<sup>11</sup> Mellanby: *Journ. of Physiol.*, xxxvi, p. 447, 1908; van Hoogenhuyse and Verploegh: *Zeitschr. f. physiol. Chem.*, lvii, p. 161, 1908.

<sup>12</sup> Shaffer: *Amer. Journ. of Physiol.*, xxiii, p. 1, 1908.

<sup>13</sup> Levene and Kristeller: *ibid.*, xxiv, p. 45, 1909.

<sup>14</sup> *Ibid.*

<sup>15</sup> Klercker: *Zeitschr. f. klin. Med.*, lxviii, p. 22, 1909; Ewing and Wolf: *Arch. of Int. Med.*, iv, p. 330, 1909; Shaffer and Coleman: *ibid.*, iv, p. 538, 1909.

<sup>16</sup> Klercker: *loc. cit.*; Wolf and Lambert: *ibid.*, v, p. 406, 1910.

<sup>17</sup> Shaffer: *loc. cit.*; Krause: *Quart. Journ. of Exp. Physiol.*, iii, p. 289, 1910; Taylor: *Biochem. Journ.*, v, p. 362, 1911.

<sup>18</sup> Underhill and Rand: *Arch. of Int. Med.*, v, p. 61, 1910.

<sup>19</sup> Shaffer: *loc. cit.*

<sup>20</sup> Murlin: *Amer. Journ. of Physiol.*, xxviii, p. 422, 1911.

<sup>21</sup> Mellanby: *Proc. Roy. Soc.*, B, lxxxvi, p. 88, 1913.

<sup>22</sup> Rose: this *Journal*, x, p. 265, 1911.

<sup>23</sup> Folin and Denis: *ibid.*, xi, p. 253, 1912.

<sup>24</sup> *Loc. cit.*

effect, they consider this a demonstration of an exogenous excretion of creatine.

That the creatine eliminated in the urine during starvation was derived from the creatine of the muscle, was the original supposition of Benedict,<sup>25</sup> though few data have been submitted definitely bearing on this point. Mendel and Rose<sup>26</sup> have recently investigated the influence of starvation upon the creatine content of muscle. They have observed a very appreciable increase in the creatine concentration of muscle during starvation in seven out of eight experiments upon rabbits, and four out of five experiments upon hens. From these observations they conclude that without doubt there occurs an increase in the percentage of creatine in the muscles of rabbits and hens during inanition. This they interpret as due to an increased creatine formation. Aside from these observations, Dorner<sup>27</sup> has reported one experiment upon a starving rabbit in which a decreased concentration of creatine was observed, while Howe and Hawk<sup>28</sup> have reported an experiment upon a starving dog in which a marked reduction in the creatine content of the muscle was found. Further discussion of this subject will be made in connection with our experiments.

In an earlier communication<sup>29</sup> attention was called to the constancy in the content of muscle creatine for normal animals of a given species, though distinctive for different animals, *e.g.*, 0.52 per cent for the rabbit, 0.37 per cent for the dog, etc. In the case of the rabbit, observations upon the creatine concentration of the muscle were reported for twenty animals, the results being very uniform and many of them identical. It was suggested that this might serve to explain the constancy in the elimination of creatinine. In twelve experiments determinations were also made of the total amount of creatine present in the tissues of the body, and the ratio which existed between this and the amount of the daily elimination of creatinine. For animals of nearly the same weight, the ratio was very constant. It is reasonable to believe that if the creatine eliminated in the urine during starvation

<sup>25</sup> *Loc. cit.*

<sup>26</sup> Mendel and Rose: *this Journal*, x, p. 255, 1911.

<sup>27</sup> *Loc. cit.*

<sup>28</sup> *Loc. cit.*

<sup>29</sup> Myers and Fine: *this Journal*, xiv, p. 9, 1913.

owes its origin to the disintegration of muscle tissue, the total creatine of the body will be depleted in proportion to the amount eliminated in the urine, provided creatine is not readily destroyed in the body. It was upon this hypothesis that the present investigation was undertaken; in fact, the data previously reported were in part carried out as a control to those reported in the present paper.

#### METHODS EMPLOYED.

In general, the methods employed were the same as those described in our previous paper. The present experiments were made upon twenty-two rabbits, eighteen of which were subjected to single periods of starvation, while the remaining four were deprived of food for two ten-day periods, a feeding period of ten days being interposed between these two periods. In all cases a control period of one week was obtained, during which the animals were fed upon a uniform diet of 350–400 grams of carrots. After the control period in the first series of experiments, the animals were starved to the point of death. The urines were collected in weekly periods, the daily samples being preserved with toluene at 0°C. during the interval. The analytical determinations in these periods included total nitrogen, creatinine and creatine. In the majority of cases the animals were killed when it was certain they would not survive more than a few hours longer. In some cases, however, the animals died unexpectedly during the night, in which event the samples of muscle and the carcass were ground up and covered with alcohol as soon as possible on the following morning. It is not believed that any very appreciable error was introduced in this way.<sup>30</sup> The carcass and

<sup>30</sup> Several experiments were made to secure light on this point. Four may be cited. A sample of muscle from rabbit 64 had a moisture content of 73.8 per cent and a creatine content of 0.534 per cent. Upon allowing a weighed sample of muscle to stand at room temperature without any preservative for two days the same content of creatine was found. A similar experiment was performed with rabbit 65 allowing the sample to stand three days with no change in the content of creatine (total creatinine). Rabbit 63 was allowed to remain at room temperature for twenty-four hours before taking the samples of muscle. The muscle was then found to have a moisture content of 76.7 per cent, a nitrogen content of 3.28 per cent, while two separate samples were both found to contain 0.516 per cent



a sample of muscle from the hind legs were analyzed for creatine as previously described. In many cases it was not possible to obtain 100 gram samples as was done with the normal animals, and in these instances 40 grams were generally taken. In addition, moisture and nitrogen determinations were made on the muscle in most cases.

The rabbit is a particularly suitable animal for the experiments described, by reason of the ease with which starvation causes an excretion of creatine, and further, because the size of the animal makes a determination of the total creatine content of the body comparatively easy.

On account of the very large amount of data, the detailed protocols are not included in this paper. The more important facts in the first series of experiments may be found in Table I, those of the second series in Table IX. Special tables have been made for the individual topics of interest.

#### EXPERIMENTAL PART.<sup>31</sup>

In the first series of eighteen rabbits with a single period of starvation, the length of life, as shown in Table I, varied between six and twenty-seven days, with an average of two weeks. The loss in weight varied from 35 to 50 per cent, depending upon the length of the fast.

##### *Influence of starvation upon the content of muscle creatine.*

As may be observed in Table I, the content of creatine in the muscle is relatively increased in a short fast, while in a long fast it is generally decreased. This decrease is, furthermore, often accentuated by the high content of moisture (about 80 per cent).

creatine. In the case of rabbit 68, an analysis of the muscle of the right leg, in which samples were taken at once after killing, yielded: moisture, 78.4 per cent, nitrogen 3.30 per cent and creatine 0.522 per cent. Twenty-four hours later, samples of muscle were removed from the other leg with the following result: moisture, 79.4 per cent, nitrogen 3.05 per cent and creatine 0.511 per cent.

<sup>31</sup> A preliminary report of these experiments was made to the Society for Experimental Biology and Medicine, October 16, 1912, see *Proceedings*, x, p. 12, 1912. We were assisted in these experiments by Mr. Adolph Bernhard.

TABLE I.  
*Influence of starvation upon the creatine content of the body—summary table.*

ANIMAL	INFLUENCE OF STARVATION UPON									
	Body weight			Composition of muscle			Creatine content of body tissues, etc.			
	Initial weight kgms.	Weight at death kgms.	Loss in weight per cent	Moisture per cent	Nitrogen per cent	Creatine per cent	Creatine ex- creted in urine grams	Creatine content of tissues at death grams	Creatine content of tissues and urine grams	Creatine of tissues and urine per cent
days	LENGTH OF STARVATION									
37	1.55	0.98	36.8	78.3	3.47	0.550	0.73	1.71	2.44	0.157
41*	2.34	1.83	21.8	75.6	3.91	0.573	0.22	3.87	4.09	0.175
17	1.91	1.21	36.7			0.587	0.79	2.61	3.40	0.170
13†	1.97	1.26	36.0			0.618	0.58	2.60	3.18	0.159
44	1.85	1.06	42.7	79.4	3.38	0.524	1.28	1.69	2.98	0.161
24†	1.64	0.91	44.5	80.2	3.22	0.380	0.91	1.27	2.18	0.133
40	1.84	1.04	43.5	79.5	3.21	0.417	2.07			
20†	1.77	1.07	39.6	76.0		0.556	0.62	2.19	2.81	0.159
21†	1.63	0.93	42.0			0.313	1.26	1.17	2.43	0.149
23	1.46	0.83	43.1	81.1		0.397	1.30	1.26	2.50	0.171
38	1.78	1.12	37.1	78.6	3.53	0.492	0.90	1.88	2.77	0.156
19†	1.81	1.09	39.7			0.397	1.24	1.70	2.95	0.163
43	1.74	0.94	46.0	77.6	3.64	0.426	1.51	1.17	2.68	0.154
42†	1.88	1.01	46.3	80.3	3.24	0.357	1.20	1.28	2.48	0.132
36†	1.79	0.95	46.9	79.1	3.30	0.402	1.55	1.51	3.06	0.171
39	2.27	1.10	51.6	80.8	3.19	0.361	1.91	1.34	3.25	0.143
14†	1.69	0.89	47.3			0.522	0.49	1.91	2.40	0.140
16††	2.33	1.19	48.9	77.6	3.70	0.382	0.89	1.91	2.80	0.120

\* Rabbit killed at the end of one week, though it obviously would have lived longer.

† Rabbit died.

†† Rabbits 14 and 16 both pregnant and aborted early in starvation, their power of resistance apparently increased.

TABLE I—Continued.

ANIMAL	CREATININE ELIMINATED IN URINE DURING STATION		CREATININE OF TISSUES AND URINE AS CREATININE IN TERMS OF INITIAL WT.		AVERAGE DAILY LOSS OF			LOSS PER KILO OF LOSS IN WEIGHT			IN PERCENTAGE OF TOTAL NITROGEN AVERAGE FIGURES				AVERAGE DAILY ELIMINATION OF CREATININE		
	grams	per cent	Weight grams	Nitrogen		Creatinine mgms.	Nitrogen grams	Creatinine N grams	Creatinine and cre- atinine N grams	Creatinine N	Creatinine N	Creatinine and cre- atinine N	mgms.	During starvation	During control mgms.	Increase or de- crease during starvation per cent	
				grams	mgms.												
37	0.35	0.171	95	1.31	122	16.1	0.41	0.61	2.6	1.2	3.8	3.8	49	55	-11		
41	0.72	0.205	73	0.86	32	11.8	0.14	0.59	1.2	3.8	5.0	5.0	88	86	+2		
17	0.55	0.197	88	1.19	99	13.7	0.36	0.61	2.7	1.8	4.5	4.5	59	71	-17		
13	0.55	0.186	79	1.16	65	14.7	0.26	0.50	1.8	1.6	3.4	3.4	51	69	-26		
44	0.68	0.198	79	1.49	128	18.9	0.52	1.01	2.8	1.5	4.3	4.3	59	62	-5		
24	0.66	0.193	66	1.33	83	20.0	0.40	0.69	2.0	1.4	3.4	3.4	51	66	-22		
40	0.93		68	1.32	124	19.7	0.60	0.97	3.0	1.9	4.9	4.9	67	63	+6		
20	0.71	0.199	82	0.80	47	14.9	0.28	0.61	1.9	2.2	4.1	4.1	47	74	-36		
21	0.86	0.201	50	0.91	90	18.1	0.58	0.77	3.2	2.2	5.4	5.4	53	77	-31		
23	0.69	0.219	44	1.17	93	26.0	0.66	1.05	2.6	1.4	4.0	4.0	42	58	-28		
38	1.26	0.224	44	1.00	60	18.0	0.44	1.05	1.9	2.7	4.6	4.6	72	49	+47		
19	1.04	0.221	73	1.09	83	22.8	0.55	1.02	2.4	2.0	4.4	4.4	60	76	-21		
43	1.18	0.222	47	1.15	63	24.5	0.60	1.08	2.5	1.9	4.4	4.4	60	66	-9		
42	1.44	0.209	46	1.01	63	22.0	0.44	0.97	2.0	2.4	4.4	4.4	66	73	-10		
36	1.33	0.245	38	0.96	71	25.2	0.59	1.11	2.4	2.0	4.4	4.4	52	65	-20		
39	1.97	0.230	40	1.04	80	21.3	0.52	1.06	2.5	2.5	5.0	5.0	71	84	-16		
14	1.43	0.224	32	0.49	18	15.3	0.18	0.71	1.2	3.4	4.6	4.6	45	61	-26		
16	1.79	0.197	42	0.69	33	16.4	0.25	0.75	1.5	3.1	4.6	4.6	57	88	-35		

The influence of starvation upon the content of muscle creatine is best shown in Table II, in which the data have been arranged in the order of creatine concentration.

TABLE II.

*Influence of starvation upon the creatine content of rabbit muscle.\**

GROUP	ANIMAL	LENGTH OF STARVATION	CREATINE CONTENT OF MUSCLE†	CREATINE ELIMINATED IN URINE DURING STARVATION	AMOUNT OF INITIAL BODY CREATINE ELIMINATED IN URINE	CREATINE UNACCOUNTED FOR
		days	per cent	grams	per cent	per cent
A.....	44	10	0.610	1.28	38	12
	37	6	0.608	0.73	26	15
	41	7	0.564	0.22	5	4
	20	13	0.556	0.62	19	13
	38	15	0.552	0.90	28	14
Average.....		10	0.578	0.75	23	12
B.....	23	14	0.504	1.30	49	8
	40	12	0.488	2.02		
	36	22	0.462	1.55	48	5
	24	11	0.461	0.91	30	24
Average.....		15	0.479	1.48	42	12
C.....	43	17	0.456	1.51	50	15
	39	24	0.451	1.91	46	21
	42	19	0.435	1.20	35	27
	16	27	0.410	0.89	21	34
Average.....		22	0.438	1.38	38	24

\* In this table, only those experiments have been included in which moisture determinations were made in the muscle.

† Figures reduced to moisture content of 76 per cent.

To make the results comparable, the figures for the concentration of creatine have been reduced to a uniform moisture content of 76 per cent, which is approximately the normal content. With one exception (Rabbit 41), all animals were allowed to come

to the point of death. A study of this table shows that in group A, in which an increase in the content of creatine was observed, the length of the fast was comparatively short, and that much less creatine was eliminated in the urine than in group B. The high content of creatine is interpreted as due to a more rapid loss of the non-creatine containing portion of the muscle than of that containing the creatine. In group B, however, an average of twice as much creatine is lost in the urine. This loss is sufficient to cause a very decided lowering of the muscle creatine, from 0.58 to 0.48 per cent. The chief factor here is obviously the marked excretion of creatine in the urine. In group C, in which the average length of life is a week greater, we find a still further depletion of the muscle creatine, 0.44 per cent, with, however, a smaller excretion of creatine in the urine. It is evident that another factor must play a part here, and an inspection of the table shows that the amount of creatine remaining unaccounted for, that is not present in the body at death or eliminated in the urine, is twice as great in this group as in groups A and B. It is possible that in animals of group C, where the length of life was the longest, the oxidative power of the body was greater and that they were able to live more economically. The possible loss of creatine by the excretion of creatinine must not be forgotten, however.

That the decreased concentration of muscle creatine is actually due to the loss of creatine in the urine may be illustrated in another way. As pointed out above, the loss of creatine during the last days of life forms a very large part of the total elimination. By adding the creatine excreted during this terminal period to the creatine still present in the body at death, one may ascertain the creatine content and concentration of the body at the stage in the fast before the excessive elimination of creatine took place. With the ratio between the percentage of creatine in the body at these two periods and the content of muscle creatine at death, the concentration of creatine at an earlier period in the fast may be ascertained.<sup>32</sup> To illustrate this point four

<sup>32</sup> The method of calculation may be illustrated in case of rabbit 23. The creatine content of the body at death was 1.261 grams and the weight 0.83 kgm., thus giving a creatine concentration of 0.152 per cent. Seven days previous to death the body weight was 1.15 kgms., while the creatine

experiments have been picked from the above groups B and C, in which considerable amounts of creatine were eliminated in the urine. As shown in Table III, the concentration of rabbit 39, which fasted twenty-four days, was 0.45 per cent at death, while on the twentieth day of starvation, according to this calculation, it would have been 0.63 per cent.

Not only do these results appear to demonstrate that the decrease in the muscle creatine is due to the loss of creatine in the

TABLE III.

*Influence of the rapid excretion of creatine upon the creatine content of muscle.*

ANIMAL	LENGTH OF STARVATION	CREATINE ELIMINATED DURING STARVATION	CREATINE ELIMINATED DURING LAST DAYS OF STARVATION	CREATINE CONTENT OF MUSCLE AT DEATH*	CALCULATED CONTENT OF MUSCLE CREATINE* AT 4-9 DAYS BEFORE DEATH
	days	grams	grams	per cent	per cent
23	14	1.30	1.10 (7 days)	0.504	0.681 on 7th day
36	22	1.55	1.07 (9 days)	0.462	0.550 on 13th day
43	17	1.51	0.89 (4 days)	0.456	0.635 on 13th day
39	24	1.91	0.95 (4 days)	0.451	0.634 on 20th day

\* Figures reduced to moisture content of 76 per cent.

urine, but they show that a very decided increase in the concentration may actually exist up to a relatively short time before death.

In their eight experiments upon starving rabbits, Mendel and Rose found that in seven instances there was a decided increase in the concentration of the creatine in the muscle. For the exception they suggest the age of the animal as a possible explanation

excreted during the last seven days of life amounted to 1.098 grams. This creatine when added to that present in the body at death gave a total of 2.359 grams, and a creatine content for the body of 0.205 per cent. The creatine content of the muscle at death, 0.504 per cent times 0.205 divided by 0.152 gave 0.681 per cent as the muscle creatine content seven days previous to death.

in view of Mellanby's<sup>33</sup> observation that young animals have much less creatine than adults. As the animal in question weighed 2.54 kgms., this would hardly appear to be a very satisfactory explanation. The uniformly high figures of Mendel and Rose may easily be explained by the results given in Table III. These authors have pointed out the great importance of ascertaining the moisture content of the muscle in starvation before drawing conclusions as to the content of creatine. That this is essential may be seen by inspecting Table I. In our earlier experiments we were primarily concerned with the determination of the total content of creatine in the body rather than that of the muscle; and on this account, we did not adequately appreciate this point, and hence in the earlier experiments moisture determinations were not always made. The moisture may make a difference as great as 20 per cent, and it is true that the results of Dorner<sup>34</sup> might have been explained on this basis. This would hardly suffice, however, to explain the results of Howe and Hawk.<sup>35</sup>

*The creatine of the urine during starvation.*

Since the discovery by Benedict of the elimination of creatine in the urine during starvation, it has generally been assumed that the creatine appearing in the urine in this condition, and also in pathological conditions associated with malnutrition and loss in weight had its origin in the muscle. So far as we are aware, this has never been conclusively demonstrated. The data which have been presented on the influence of creatine in the urine upon the content of creatine in the muscle point very clearly to the generally assumed origin. The fact that the creatine eliminated in the urine during the period of starvation accounts for the reduction in the creatine content of the body as shown in Tables I, III, and IV very greatly strengthen this view. About 12 per cent still remains unaccounted for in the fast of ordinary length, but this is what we might expect from our knowledge of

<sup>33</sup> *Loc. cit.*

<sup>34</sup> *Loc. cit.*

<sup>35</sup> *Loc. cit.*, see also paper by Biddle and Howe: *Biochem. Bull.*, ii, p. 386, 1913, which appeared after the present paper had been submitted for publication.

the fate of administered creatine. It is possible that this is due to the excess of creatine destruction over the creatine formation.

As shown in Table V and briefly summarized in Table VI below, the urinary excretion of creatine rises very rapidly in the last days of life. Animals which live for only a short period excrete relatively larger amounts the first week than those which

TABLE IV.

*Influence of starvation upon the creatine content of the body.*

ANIMAL	LENGTH OF STARVATION	INITIAL CONTENT OF CREATINE IN BODY, CALCULATED FROM INITIAL WT. $\times 0.182^*$	RELATION TO INITIAL CREATINE			IN PER CENT OF INITIAL CREATINE		
			Creatine of body at death	Creatine eliminated in urine	Creatine unaccounted for	Creatine of body	Creatine of urine	Creatine unaccounted for
	days	grams	grams	grams	grams	per cent	per cent	per cent
37	6	2.82	1.71	0.73	0.38	59	26	15
41	7	4.26	3.87	0.22	0.17	91	5	4
17	8	3.48	2.61	0.79	0.08	75	23	2
13	9	3.59	2.60	0.58	0.41	73	16	11
44	10	3.37	1.69	1.28	0.44	50	38	12
24	11	2.99	1.27	0.91	0.81	46	30	24
40	12	3.35		2.07			62	
20	13	3.22	2.19	0.62	0.41	68	19	13
21	14	2.97	1.17	1.26	0.54	40	42	18
23	14	2.66	1.26	1.30	0.10	53	49	8
38	15	3.24	1.88	0.90	0.46	58	28	14
19	15	3.29	1.70	1.24	0.35	51	38	11
43	17	3.17	1.17	1.51	0.49	35	50	15
42	19	3.42	1.28	1.20	0.94	38	35	27
36	22	3.26	1.51	1.55	0.20	47	48	5
39	24	4.13	1.34	1.91	0.88	33	46	21
14	25	3.08	1.91	0.49	0.68	62	16	22
16	27	4.26	1.91	0.89	1.44	55	21	34

\* Calculated from average data in Table VII of a previous paper, this *Journal*, xiv, p. 23, 1912.

live for a much longer period. As shown in table VI, animals living two weeks eliminated two-thirds of the total creatine excreted during the second week; those living three weeks, one-half during the last week; while those which lived close to four weeks eliminated one-third the last week. The gradual rise in the elimination of creatine was brought out very nicely in one of Benedict's earlier experiments.



TABLE V.  
*Rate of creatine excretion during starvation.*

ANIMAL	LENGTH OF STARVATION	TOTAL CREATINE EXCRETION	RATE OF CREATINE EXCRETION DURING STARVATION							
			First week		Second week		Third week		Fourth week	
			gram	per cent	grams	per cent	gram	per cent	gram	per cent
37	6	0.73	0.73	100						
41	7	0.22	0.22	100						
17	8	0.79	0.79	100						
13	9	0.58	0.58	100						
44	10	1.28	0.57	44	0.71	56				
24	11	0.91	0.68	75	0.23	25				
40	12	2.07	0.59	28	1.49	72				
20	13	0.62	0.14	23	0.48	77				
21	14	1.26	0.43	34	0.83	66				
23	14	1.30	0.20	15	1.10	85				
38	15	0.90	0.26	29	0.64	71				
19	15	1.24	0.24	19	1.00	81				
43	17	1.51	0.17	11	0.69	46	0.66	43		
42	19	1.20	0.21	18	0.44	37	0.56	45		
36	22	1.55	0.23	15	0.35	23	0.97	62		
39	24	1.91	0.41	21	0.25	13	0.55	29	0.71	37
14	25	0.49	0.14	29	0.14	29	0.13	26	0.08	16
16	27	0.89	0.28	31	0.17	19	0.13	15	0.31	35

TABLE VI.  
*Average data on rate of creatine excretion during starvation.*

NUMBER OF ANIMALS	LENGTH OF LIFE	CREATINE EXCRETED DURING STARVATION	CREATINE EXCRETED DURING LAST WEEK OF LIFE	AMOUNT OF CREATINE EXCRETION IN WEEKLY PERIODS			
				First week	Second week	Third week	Fourth week
	weeks	grams	per cent	gram	gram	gram	gram
3*	1	0.70	100	0.70			
8	2	1.20	68	0.39	0.81		
3	3	1.42	51	0.20	0.49	0.73	
3	4	1.10	34	0.27	0.19	0.27	0.37

\* Does not include Rabbit 41 which was killed at the end of one week, but obviously long before it would have died.

*Possible relation of creatine to creatinine.*

That the elimination of creatinine falls slightly during the period of starvation has, in general, been the conclusion of the various investigators who have studied this problem. In some cases, however, the fall has been so slight as to be almost negligible. The average daily elimination for week periods in our series of eighteen experiments is given in Table VII. In some

TABLE VII.  
*Average daily elimination of creatinine—weekly periods.*

ANIMAL	CONTROL WEEK	STARVATION			
		First week	Second week	Third week	Fourth week
	mgms.	mgms.	mgms.	mgms.	mgms.
37	55	50			
41	86	88			
17	71	59			
13	69	60	22		
44	62	82	61		
24	66	58	40		
40	63	72	60		
20	74	43	53		
21	77	63	42		
23	58	47	38		
38	49	48	81		
19	76	37	80		
43	66	66	54	58	
42	73	75	65	52	
36	65	58	54	45	
39	84	92	70	57	54
14	61	51	49	32	42
16	88	62	62	44	60

cases there appeared to be a slight rise in the elimination of creatinine during the first week of starvation, while in a number of cases there was a higher elimination during the last week of life than during the preceding week. In general, however, there was a decrease. Since the weight of the whole series of animals was quite uniform, the average data may be of interest. The average daily elimination of creatinine during the control period was 70 mgms. for the eighteen animals; for the first week of starva-

tion, 62 mgms.; for the second week, 55 mgms.; for the third, 48 mgms., and for the fourth week, 52 mgms. This fall in the excretion of creatinine was shown especially well in the experiments of Mendel and Rose.

If any relation exists between the creatine of the muscle and the creatinine of the urine, and if the suggestion made in the first paper of this series—that the constancy in the content of creatine in the normal animal affords a possible explanation for the constancy in the excretion of creatinine—is correct, we would expect that a decreased excretion of creatinine would be preceded by a lowering of the creatine storehouse. In reality such is found to be the case. Since the elimination of creatine in the urine is not excessive until the days immediately preceding death, the creatine content of the body is not markedly lowered until that time. For the series of eighteen animals, the average amount of creatine found in the body at death amounts to about 55 per cent of the original content. In other words, the total loss amounts to about 45 per cent. Since the greater part of this is lost during the last days of life, the depletion is probably not much above 15 to 20 per cent during the greater part of the starvation, and this corresponds in a general way to the decrease in the excretion of creatinine. This idea was previously suggested by one of us<sup>36</sup> as a possible explanation of the decreased excretion of creatinine in pathological conditions associated with an excretion of creatine. Furthermore, it would appear evident from the recent investigations of Chisolm<sup>37</sup> that in these same conditions there was a decrease in the creatine content of the muscle. Further analyses of human muscle appear particularly desirable, especially those associated with an excretion of creatine.

It seemed important to ascertain if the creatinine eliminated during starvation would account for the creatine not present in the body at death or previously eliminated in the urine, *i.e.*, the creatine which has been tabulated as unaccounted for. Such data are given in Table I. During a long period of starvation, the creatinine calculated as creatine is considerably in excess (about 20 per cent) of the creatine which remains unaccounted for. Although this excess may represent the rate of creatine-

<sup>36</sup> Myers: *Amer. Journ. of Med. Sci.*, cxxxix, p. 256, 1910.

<sup>37</sup> Chisolm: *Biochem. Journ.*, vi, p. 243, 1912.

creatinine formation, obviously no conclusions can be drawn in light of our present lack of knowledge of the subject. In our previous communication this point was mentioned, and it was hoped that this might throw some light on the relation of creatine to creatinine; but the only relationship we have been able to elicit is that brought out in the two paragraphs below.

In their paper Mendel and Rose<sup>38</sup> pointed out that although there was no apparent relation between the creatine nitrogen or creatinine nitrogen and the total nitrogen, nevertheless, the "total creatinine" nitrogen and the total nitrogen did appear to run parallel. Our own results point to a similar parallelism as is shown in Table I. The creatine and creatinine nitrogen forms a very uniform proportion of the total nitrogen in the whole series of experiments.

Howe and Hawk<sup>39</sup> have shown that the loss of muscle tissue calculated from the excretion of creatine only accounts for about half that calculated from the total nitrogen eliminated. Benedict,<sup>40</sup> in discussing this topic in his original paper on the subject, pointed out that the creatine only accounted for a part of the muscle loss. Mendel and Rose have dwelt upon this point and shown that the creatinine nitrogen may in part account for this discrepancy. In Table VIII, we have calculated the average daily loss of moist muscular tissue upon the basis of the total nitrogen, the urinary creatine, and also of the urinary creatine and creatinine (in terms of creatine). The agreement between the muscle calculated from this latter source and the total nitrogen are surprisingly close, and it would seem hard to reconcile this with any other idea than that they were both closely related products of muscular metabolism.

#### *Repeated starvation.*

The results which have been obtained from the four experiments in which rabbits were starved for two ten-day periods with a feeding period of a similar length of time intervening, simply bear out the observations of the previous series of experi-

<sup>38</sup> *Loc. cit.*

<sup>39</sup> *Loc. cit.*

<sup>40</sup> *Loc. cit.*

ments. It was planned to kill two of the animals at the end of a third period of starvation, and the other two after they had been brought back to original weight from a similar length of starvation, and then ascertain the creatine concentration of the muscle and of the body. As may be observed from an inspection

TABLE VIII.

*Flesh catabolized as calculated from the elimination of nitrogen, creatine and creatinine.*

ANIMAL	FLESH CATABOLIZED CALCULATED FROM AVERAGE DAILY ELIMINATION OF		
	Total N	Creatine and creatinine as creatine	Creatine
	$N \times 28^*$	$\times 192^*$	$\times 192$
	grams	grams	grams
37	36.7	34.4	23.4
41	24.1	25.7	6.1
17	33.3	32.1	19.0
13	32.5	23.8	12.5
44	41.7	37.6	24.6
24	37.3	27.3	16.0
40	37.0	38.8	23.7
20	22.4	19.6	9.0
21	25.5	29.0	17.3
23	32.8	27.3	17.9
38	28.0	27.6	11.5
19	30.5	29.4	15.9
43	32.2	25.5	12.1
42	28.3	26.9	12.1
36	26.9	25.2	13.7
39	29.1	31.1	15.4
14	13.7	13.4	3.5
16	19.3	19.0	6.3

\* Calculated on the basis of rabbit muscle containing 3.6 per cent nitrogen and 0.52 per cent creatine.

tion of Table IX, three of the animals did not bear feeding after the second period of starvation. Rabbit 52 suffered from severe diarrhea, and, though it ate well, lost weight and died at the end of a week. In rabbits 48, 50, and 52, the concentration of creatine was not markedly below normal, though in rabbit 51,

which was killed nearly ten weeks after the last fast, the creatine concentration had not been restored to the normal, and, in fact, was very much lower than in the other three instances. This fact is very interesting and points to the difficulty with which creatine is replaced, at least on a carrot diet. In this series of experiments, the correspondence between the creatine excreted in the urine and that remaining in the tissues at death is brought out very nicely.

TABLE IX.

*Influence of repeated starvation on creatine content of body.*

ANIMAL	TOTAL LENGTH OF STARVATION	BODY WEIGHT						TIME OF DEATH AFTER LAST STARVATION
		Body weight during control period	Weight after 1st 10 days star- vation	Weight after 10 days' feeding	Weight after 2d 10 days' star- vation	Weight at death	Loss in weight at end of 2d starvation	
	days	kgms.	kgms.	kgms.	kgms.	kgms.	per cent	
48	20	1.76	1.39	1.59	0.97	0.99	45	1 day
50	20	1.70	1.28	1.63	0.90	0.87	47	1 day
51	20	1.72	1.32	1.58	1.12	1.43	35	74 days, killed
52	20	1.82	1.40	1.68	1.19	1.02	35	8 days

TABLE IX—CONCLUDED.

ANIMAL	MUSCLE					CREATINE CONTENT OF BODY				
	Molature	Nitrogen	Creatine	Creatine*	Initial creatine Content Body weight X 0.182	Creatine of tissues at death	Creatine eliminated in urine	Creatinine of urine 1st 10 days	Creatinine of urine 2d 10 days and to death	Creatine unaccounted for
	per cent	per cent	per cent	per cent	grams	grams	grams	gram	grams	gram
48	76.2	3.96	0.496	0.500	3.20	1.44	1.48	0.65	0.83	0.28
50	76.8	3.70	0.451	0.487	3.09	1.56	1.34	0.57	0.77	0.19
51	76.3		0.427	0.433	3.13	2.25	0.90	0.41	0.49	0
52	82.5	2.84	0.354	0.486	3.31	0.96	1.70	0.51	1.19†	0.65

\* Figures reduced to 76 per cent molature.

† 0.79 gram of this eliminated during last 8 days of life.

## DISCUSSION.

The experiments above described indicate that the creatine concentration of the muscle in the rabbit is first increased during starvation, then subsequently decreased. The increase is apparently due to the removal of the non-creatine portion, *e.g.*, glycogen, fat, etc., of the muscle more rapidly than that containing the creatine. The elimination of creatine in the urine increases with the length of the fast and in the days preceding death relatively large amounts are eliminated. This results in a depletion of the creatine supply of the body and in a decreased content of muscle creatine. That this is the case may be seen by inspecting the data given in Table III. The amount of creatine lost in the urine during the last four days of life may be sufficient to cause the creatine content of the muscle to drop from 0.63 to 0.45 per cent, though the creatine content of the muscle may be as high as 0.63 per cent on the twentieth day of fasting. It is believed that this explains the uniformly high results of Mendel and Rose. In animals fasting for a considerable period, three to four weeks (Table II), there is a loss of creatine which is not so well accounted for by the creatine eliminated in the urine. Possibly the oxidative powers of the body are a little greater, and this still further depletes the creatine of the muscle. The loss of creatine in the urine is still, however, the most important factor in causing a depletion in the creatine content of the muscle.

The fact that the creatine content of the muscle is so clearly dependent upon the amount and rate of creatine excretion in the urine, is very convincing proof of the origin of the creatine of the urine. When this is added to the fact that the creatine eliminated in the urine—often 40 to 50 per cent of the initial content—very largely accounts for the depletion in the creatine content of the body caused by starvation (Table IV), there would appear to be little doubt as to the source of the urinary creatine. Furthermore, the elimination of creatine is always the greatest during the last days of life, when the greatest destruction of muscle tissue, as evidenced by the increased excretion of nitrogen, occurs.

As shown in Table VI, the rate and amount of creatine excreted during starvation are determined to a considerable extent

by the length of life of the animal, which in turn is probably dependent upon its nutritive condition. For animals living two weeks, two-thirds of the total creatine eliminated is excreted the second week; for animals living three weeks, one-half the third week; while animals living four weeks eliminate one-third the fourth week.

In light of the above data, we believe there can be little doubt as to the origin of urinary creatine in pathological conditions associated with malnutrition and loss in weight, especially in view of the observations of Chisolm that there is a decrease in the content of muscle creatine, at least in some of these conditions. We have been hoping to verify Chisolm's observations in this regard, but as yet have been unable to secure sufficient material.

The excretion of creatine by growing animals and during lactation in women cannot entirely be reconciled with the above. That children eliminate creatine we have been able to verify. We hope to make the elimination of creatine by children and by nursing women the topic of a subsequent communication.

The evidence which has been thus far presented to show a relationship between creatine and creatinine is still unsatisfactory, but the data upon which several workers have declared the independence of these substances in metabolism are even more scanty. It has been stated that the only relationship which has been shown to exist is a chemical one.

In the previous paper of this series, it was pointed out that the creatine content of the muscle for a given species is remarkably constant, and it was suggested that this might be the underlying factor in the constant excretion of creatinine. Furthermore, it was shown that for rabbits of a fairly uniform body weight, the ratio which existed between the total creatine of the body and the average excretion of creatinine was very constant. It was further noted that animals having a high content of muscle creatine eliminated a correspondingly large amount of creatinine. A small amount of evidence bearing upon this question has been adduced in the present paper. It has been pointed out that the elimination of creatinine gradually decreases as starvation progresses—and this has been the observation of other workers—and further, that this decreased excretion of creatinine follows in



a general way the decline in the creatine content of the body. In Table I, figures representing average data for the whole period of starvation are given which show that whereas neither the creatine nitrogen nor the creatinine nitrogen forms any very uniform part of the total nitrogen of the urine, nevertheless the nitrogen from both of these sources, when taken together, makes a very uniform part of the total.

Various workers have shown that the muscle disintegration as calculated upon the creatine elimination only accounts for about half that figured upon the basis of the total nitrogen. Mendel and Rose have suggested that the creatinine nitrogen may in part account for this discrepancy. Data bearing on this point are given in Table VIII. With a few unexplainable exceptions, the average daily loss in muscle tissue, as calculated upon the basis of the total nitrogen and that of the creatine and creatinine (as creatine) are surprisingly close. From these data *alone* it would appear that both creatine and creatinine had a common origin, viz., in the muscle tissue.

It has been stated that since it is so difficult to completely convert creatine to creatinine or creatinine to creatine outside the body, it is illogical to believe such a conversion possible in the body. It must be admitted by the supporters of this idea, however, that it is very easy to produce a slight conversion in either direction *in vitro*; in fact, unless special precautions are taken, it is difficult to prevent a slight conversion, e.g., of creatine to creatinine. This slight conversion is quite sufficient to explain the origin of creatinine. After the administration of creatine to man, Folin<sup>41</sup> did not detect an appreciable change in the elimination of creatinine, but van Hoogenhuyze and Verploegh,<sup>42</sup> Towles and Voegtlin<sup>43</sup> and the present writers<sup>44</sup> have observed an appreciable increase in the excretion of creatinine. In our experiments, this averaged about 3 per cent.<sup>45</sup> It is well known that the crea-

<sup>41</sup> Folin: *Hammarsten's Festschrift.*, III, 1906.

<sup>42</sup> van Hoogenhuyze and Verploegh: *Zeitschr. f. physiol. Chem.*, lvii, p. 131, 1906; also Pekelharing and van Hoogenhuyze: *ibid.*, lxix, p. 407, 1910.

<sup>43</sup> Towles and Voegtlin: this *Journal*, x, p. 479, 1912.

<sup>44</sup> Paper to appear shortly.

<sup>45</sup> Since the present paper was written, Prof. S. R. Benedict has informed us that he has obtained similar results in experiments on dogs, the details of which are to appear at an early date.

tine of the muscle is only in very loose combination, as evidenced by the ease with which it may be extracted with water. Shaffer and Reinoso<sup>46</sup> have stated that muscle does contain small amounts of creatinine, 1 to 6 mgms. per 100 grams of dog muscle, amounts which they believe sufficient to account for the amount of creatinine excreted in twenty-four hours. We have made similar observations with methods which we believe preclude any conversion of creatine to creatinine. According to our figures, about 6 mgms. creatinine are present in 100 grams of fresh rabbit muscle, about one-hundredth as much creatinine as creatine. This is sufficient to account for 35 mgms. of creatinine on the basis of a body creatine content of 3.5 grams or about half the daily elimination of creatinine. Since, as already noted, creatine is only in very loose combination in the muscle, it is not difficult to believe that it is acted upon in the same way as creatine administered subcutaneously. A conversion of between 2 and 3 per cent (2.8 per cent for five rabbits with a uniform creatine-creatinine ratio) per day would entirely account for all the urinary creatinine. We have been able to demonstrate that an enzyme capable of producing this change is present in the muscle. Further discussion of this subject will be taken up in a subsequent paper.

#### CONCLUSIONS AND SUMMARY.

The creatine content of rabbit muscle is relatively increased in the early part of starvation, but decreased at the close of the starvation, owing to the great loss of creatine in the urine during this period.

The creatine appearing in the urine during starvation is derived from the muscle tissue, and there appears to be little doubt that this is true in pathological conditions associated with malnutrition and loss in weight.

The question of the origin of creatinine is discussed. It is believed that the evidence to support the contention that creatine and creatinine are independent in metabolism is entirely inconclusive, while the observations lending support to the older idea of the origin of the urinary creatinine from the muscle creatine are quite numerous, though as yet not entirely complete.

<sup>46</sup> Shaffer and Reinoso: Proc. Soc. of Biol. Chem., this *Journal*, vii, p. xxx, 1910.

## THE INFLUENCE OF CARBOHYDRATE FEEDING UPON THE CREATINE CONTENT OF MUSCLE.

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It has been shown by Cathcart<sup>1</sup> in experiments on men, by Mendel and Rose<sup>2</sup> in experiments on rabbits, and by one of us<sup>3</sup> in experiments on dogs, that the elimination of creatine during inanition can be reduced to a negligible quantity by the administration of carbohydrate. On the basis of this observation, Cathcart and Taylor,<sup>4</sup> Wolf and Osterberg,<sup>5</sup> and Mendel and Rose<sup>6</sup> have found that depriving the body tissues of carbohydrate with the aid of phlorhizin in experiments on dogs will bring about an excretion of creatine. The action of carbohydrate in this connection was previously recognized by Shaffer and Coleman,<sup>7</sup> who pointed out the value of diets high in caloric value and especially rich in carbohydrate in the treatment of human typhoid fever. They showed that in this way the loss of creatine could either be entirely prevented or reduced to a minimum, and for the reason that the body protein was spared by the carbohydrate.

Cathcart<sup>8</sup> believes that the appearance of creatine in the urine is more or less intimately connected with disturbances in the

<sup>1</sup> Cathcart: *Journ. of Physiol.*, xxxix, p. 311, 1909.

<sup>2</sup> Mendel and Rose: *this Journal*, x, p. 213, 1911.

<sup>3</sup> A study of the influence of carbohydrate (also of fat and protein) upon the excretion of creatine by starving dogs was suggested to one of us by Professor Mendel in 1907. Several experiments were carried out in the Sheffield Laboratory, though the details have never been published (see Myers: *Amer. Journ. of Med. Sci.*, cxxxix, p. 256, 1910).

<sup>4</sup> Cathcart and Taylor: *Journ. of Physiol.*, xli, p. 276, 1910.

<sup>5</sup> Wolf and Osterberg: *Amer. Journ. of Physiol.*, xxviii, p. 71, 1911.

<sup>6</sup> *Loc. cit.*

<sup>7</sup> Shaffer and Coleman: *Arch. of Int. Med.*, iv, p. 538, 1909.

<sup>8</sup> *Loc. cit.*

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metabolism of carbohydrate, while Mendel and Rose<sup>9</sup> state that without question the metabolism of creatine is intimately associated with carbohydrate metabolism, but conclude that it is difficult to form any chemical picture of the influence carbohydrate may have in preventing the excretion of creatine.

It occurred to the writers that a study of the creatine content of the muscle in animals fed upon carbohydrate for a considerable period might throw light on this interesting problem. The methods employed were the same as those described in the preceding paper.<sup>10</sup>

### EXPERIMENTAL PART.<sup>11</sup>

Five experiments are reported on animals fed almost exclusively on carbohydrate for from four to fifty-three days, and one experiment on an animal living twenty-nine days, to which creatine was given in addition to the carbohydrate. The carbohydrates employed were principally soluble starch and cane sugar. They were administered in solution with the aid of a urinary catheter employed as a stomach tube. In some cases arrowroot starch was employed to a small extent, while in an endeavor to prevent diarrhea, agar agar, protein-free milk, filter-paper and bone ash were used to a limited extent during the latter part of the feeding. These measures, however, were not efficacious in preventing diarrhea. Rabbit 46, which lived for fifty-three days, was free from diarrhea until the last three days of life. Occasionally, small amounts of carrots were given, though the amount of nitrogen administered in this way was negligible.

A summary of the results of these experiments may be found in Table I, although the results of one experiment, rabbit 46, have been tabulated in greater detail in Table III. Experiment 57 will be discussed more fully in a subsequent paper dealing with the fate of administered creatine.

<sup>9</sup> *Loc. cit.*

<sup>10</sup> Myers and Fine: this *Journal*, xv, p. 283, 1913.

<sup>11</sup> A preliminary report of these experiments was presented to the Society for Experimental Biology and Medicine, May 21, 1913; cf. Fine and Myers: *Proceedings*, x, p. 168, 1913. We were assisted in these experiments by Mr. Adolph Bernhard.

From an inspection of Table I it will be seen that the loss in weight was much less in comparison with the length of life than in the starving animals. It is further worthy of note that the muscle had a much more normal appearance than in the case of starving animals, although in this case the extracts for the creatine estimation were practically colorless, in contrast to the light

TABLE I.

*Influence of carbohydrate feeding upon the creatine content of the body.*

ANIMAL	LENGTH OF CARBOHYDRATE FEEDING	BODY WEIGHT			COMPOSITION OF MUSCLE				CREATINE OF BODY AT DEATH	
		Initial Weight	Weight at death	Loss in weight	Moisture	Nitrogen	Creatine	Creatine*		
	days	kgms.	kgms.	per cent	per cent	per cent	per cent	per cent	grams	per cent
46	53	1.65	1.16	30	77.6	3.11	0.339	0.364	1.62	0.140
53	24	2.44	1.59	35	77.8	3.49	0.367	0.397	2.51	0.158
54	19	2.12	1.60	25	78.4	3.16	0.373	0.414	2.51	0.157
55	11	1.82	1.34	26	74.3	3.94	0.596	0.556	2.76	0.206
61	4	2.22	1.90	14	71.0	4.88	0.643	0.530	3.46	0.182
57	29	1.98	1.22	38	76.2	3.55	0.482	0.486	2.18	0.179

\* Figures reduced to moisture content of 76 per cent.

TABLE I—CONCLUDED.

ANIMAL	INITIAL CONTENT OF BODY CREATINE CALCULATED IN FINAL WT. X 0.182	RELATION TO INITIAL CREATINE			CREATININE ELIMINATED DURING CARBOHYDRATE FEEDING TERMS CREATINE	IN PER CENT OF INITIAL CREATINE		
		Creatine content of body at death	Creatine excreted in urine	Creatine unaccounted for		Creatine of body at death	Creatine excreted in urine	Creatine unaccounted for
	grams	grams	grams	grams	grams	per cent	per cent	per cent
46	2.97	1.62	0.44	0.91	3.11	55	15	30
53	4.44	2.51	0.82	1.11	2.34	57	18	25
54	3.86	2.51	0.23	1.12	1.21	65	6	29
55	3.31	2.76	0.10	0.45	0.67	83	3	14
61	4.04	3.46	0.06	0.52	0.29	86	1	13
57*	2.49	2.18	0.29	0.02	2.20	88	11	1

\* 1.08 grams creatine given subcutaneously in equal doses over a period of 12 days; 60 per cent recovered in urine.

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yellow color observed in normal and starving animals. An appreciable amount of creatine was eliminated in the urine, though in some of the experiments the greater part of this was eliminated during the last days of life.

In general, the influence of the carbohydrate feeding upon the creatine content of the muscle was very similar to that observed in the starving animals. In animals living for only a short period there was an actual increase in the creatine content of the muscle, but with the increase in the length of the period there was a decreased concentration. When the carbohydrate feeding extended to three weeks or over, the creatine concentration was lower than that observed during starvation, although the absolute

TABLE II.  
*Creatine content of muscle as influenced by starvation and carbohydrate feeding.*

AVERAGE DATA FROM RABBITS	LENGTH OF STAR- VATION OR CARBOHYDRATE FEEDING	LOSS IN BODY WEIGHT	CREATINE CONTENT OF MUSCLE†	CREATINE ELIMINATED IN URINE	AMOUNT OF INITIAL BODY CREATINE ELIMINATED IN URINE	CREATINE UNACCOUNTED FOR
	days	per cent	per cent	grams	per cent	per cent
44, 37, 41, 20, 38*	10	35	0.578	0.75	23	12
23, 40, 36, 24*	15	45	0.479	1.48	42	12
43, 39, 42, 16*	22	48	0.438	1.38	38	24
46, 53, 54	32	30	0.392	0.50	13	28

\* See Table II, preceding paper.

† Figures reduced to moisture content of 76 per cent.

amount of creatine present in the body at death was greater than in the starvation experiments. That such would be the case might have been inferred from the smaller percentage loss in weight.

A comparison of the results of three of the experiments, namely those on the animals living three weeks or over, with the starvation experiments is shown in Table II. The influence of the different factors previously mentioned is well brought out in this table. It would appear that the action of the carbohydrate in inhibiting the elimination of creatine, was dependent in large part upon the sparing action of the carbohydrate upon the body pro-

TABLE III.  
Protocol table—carbohydrate rabbit 46.

DATE WEEK PERIODS 1912-1913	BODY WEIGHT AT END OF PERIODS	AVERAGE DAILY DIET				CREATININE		TOTAL N DAILY AVERAGE	IN PER CENT OF TOTAL N		
		Carrots	Starch or wheat starch	Sucrose	Water	Per week	Daily average		Creatinine N	Creatinine N	Creatinine and creatine N
		grams	grams	grams	grams	grams	mgms.		per cent	per cent	per cent
Dec. 24-31.....	1.63	350				0.359	51	0.66	2.9	0	2.9
Dec. 31-Jan. 7.....	1.45	50	10	5	30	0.335	48	0.40	4.5	0.7	5.2
Jan. 7-Jan. 14.....	1.44	20	15	10	50	0.382	55	0.18	11.4	1.3	12.7
Jan. 14-Jan. 21.....	1.39	15	5	10	50	0.386	55	0.22	9.3	1.6	10.9
Jan. 21-Jan. 28.....	1.35	5	15	10	50	0.358	51	0.24	7.9	0.5	8.4
Jan. 28-Feb. 4.....	1.30	20	0	10	60	0.354	51	0.29	6.5	0.7	7.2
Feb. 4-Feb. 11.....	1.23	0	5	15	70	0.307	44	0.08	20.5	2.0	22.5
Feb. 11-Feb. 18.....	1.19	0	10	15	70	0.343	49	0.13	14.0	2.0	16.0
Feb. 18-Feb. 22.....	1.20	0	10	20	90	0.159	39	0.17	8.5	3.8	12.3
	1.16										

\* Urine during last few hours of life (10 cc.) contained 25 mgms. of creatine but no creatinine.

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tein, *i.e.*, the muscle tissue, in consequence of which the muscle disintegration was much slower than in starvation.

From the creatine content of the muscle of rabbit 57, to which creatine was given subcutaneously, it would appear that the creatine of the muscle had been partially protected or possibly replaced by the administered creatine. This is further borne out by the relatively large percentage of creatine found in the body at death.

The results of the urine analyses have been tabulated in weekly periods (Table III) in the case of rabbit 46. In this experiment the amount of carrots given during the period of carbohydrate feeding was a little greater (total of 750 grams) than in the subsequent experiments. A gram and a half of nitrogen over a period of fifty-three days, however, would be a relatively unimportant consideration. The carbohydrate intake was insufficient in this case to prevent the elimination of creatine, though its excretion was restricted to a comparatively small amount, and remained fairly constant until shortly before death. The excretion of creatinine was very constant, there being comparatively little fall until the last days of life.

### CONCLUSIONS.

The influence of carbohydrate feeding upon the creatine content of rabbit muscle is similar to that observed in starvation, although after a long period of feeding there may be an even greater reduction in the creatine concentration.

The decreased elimination of creatine after feeding carbohydrate is primarily dependent upon the sparing action of carbohydrate upon the muscle protein, or, in other words, is simply one phase of the sparing action of carbohydrate on protein metabolism.



## THE RELATION OF GROWTH TO THE CHEMICAL CONSTITUENTS OF THE DIET.<sup>1</sup>

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Our earlier experiments, undertaken to determine the relative value of different purified proteins in nutrition, revealed the fact that *growth* depends on nutritive conditions which are distinct from those required for maintenance.<sup>2</sup> This fact has since been confirmed by the experimental work of others, notably McCollum,<sup>3</sup> and Hopkins.<sup>4</sup> Some of the viewpoints in respect thereto have been discussed by us elsewhere.<sup>2</sup> In addition to our earlier experience with rats, the dissimilarity in the nutritive requirements of maintenance and growth have more recently been clearly manifested in experiments on mice conducted by Dr. Ruth Wheeler in our laboratories.<sup>5</sup>

<sup>1</sup> The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

<sup>2</sup> Osborne and Mendel: Feeding Experiments with Isolated Food-Substances, Carnegie Institution of Washington, Publication 156, Parts I and II, 1911; The Rôle of Different Proteins in Nutrition and Growth, *Science*, xxxiv, pp. 722-732, 1911; Beobachtungen über Wachstum bei Fütterungsversuchen mit isolierten Nahrungssubstanzen, *Zeitschr. f. physiol. Chem.*, lxxx, pp. 307-370, 1912; The Rôle of Gliadin in Nutrition, this *Journal*, xii, pp. 473-510, 1912; Maintenance Experiments with Isolated Proteins, *Ibid.*, xiii, pp. 233-276.

<sup>3</sup> McCollum, E. V.: The Nature of the Repair Processes in Protein Metabolism, *Amer. Journ. of Physiol.*, xxix, pp. 215-237, 1911.

<sup>4</sup> Hopkins, F. G.: Feeding Experiments Illustrating the Importance of Accessory Factors in Normal Dietaries, *Journ. of Physiol.*, xlv, pp. 425-459, 1912.

<sup>5</sup> Wheeler, Ruth: *Journ. of Exp. Zool.*, 1913 (in press).

These well verified facts serve to raise the question as to what is the factor in the diet which is peculiarly essential for growth. Our own experiments, as well as those of the other investigators mentioned, make it clear that something further than a sufficient supply of energy-yielding food material is required to promote a normal growth. The animal cells need for their activities not only energy, but also suitable constructive material to replace the wear-and-tear therein. Furthermore the cells are concerned in the elaboration of a great diversity of complex and little understood substances such as enzymes, products of internal secretion, etc., which unquestionably play an indispensable rôle in life and may require either special antecedent products for their construction, chemical activators of some sort, or minute quantities of readily overlooked rarer elements and compounds. It is easy, yet futile at the present time, to develop detailed hypotheses respecting the almost innumerable possibilities involved. The greatest promise of success in discovering the food factors which determine successful growth lies in seeking them in some chemical constituents of such diets as have proved adequate to promote growth.

In attempting to ascertain what constitutes an adequate diet, by feeding experiments with isolated substances, we have found that our purely artificial foods—mixtures of isolated proteins, fats, carbohydrates and inorganic salts—sooner or later fail to maintain mature animals. In view of this such dietaries may naturally be expected to fail to maintain the less resistant young during their adolescent period. It is true that in several instances we have succeeded in keeping grown rats in health and in apparent nutritive equilibrium on purely artificial food mixtures over periods far longer than the experience of our predecessors had led us to expect. But the outcome has never been satisfactory in the sense of extending over what may be considered as the larger portion of the life span of an adult animal. Successful maintenance has been secured only when the animals were fed, in part at least, with foods containing our "protein-free milk," the preparation and composition of which has been detailed elsewhere.<sup>6</sup> The superiority of the latter foods, compared with any

<sup>6</sup> Osborne and Mendel: Feeding Experiments with Isolated Food-Substances, Carnegie Institution of Washington, Publication 156, 1911, Part II, p. 80.

purely artificial food mixture in repairing the depleted body weight of animals that have begun to decline on the artificial salt mixtures tested is beyond question. Instances of immediate recovery following the replacement of the inorganic constituents of the dietaries and part of the carbohydrates by the "protein-free milk" have been published, and might be duplicated in great numbers from our protocols.<sup>7</sup> Even greater success is manifested in maintenance experiments in which the "protein-free milk" alone furnished the inorganic constituents of the dietary during long periods of time.<sup>8</sup> The superiority of the "protein-free milk" foods over the diets containing artificial salt mixtures in the maintenance experiments also is unquestioned. Wherein the difference lies is not yet apparent. We have already pointed out that the efficiency of this adjuvant to the energy-yielding nutrients is not attributable to the minute trace of milk protein present. The fact of the greater efficiency of the natural milk product suggests that some constituent present in milk is essential for prolonged maintenance.

In our numerous experiments milk has proved to be an adequate food, both for growth and maintenance. Young rats fed solely upon the milk food which we have been accustomed to use<sup>9</sup> not only have grown from infancy to full maturity, but have also given birth to litters of normal young which in turn have thriven on diets precisely like that furnished to their parents, as illustrated by chart I in the appendix. One must conclude from these facts that the milk food contains all that is essential for both growth and maintenance.

We have imitated the gross composition of this highly successful milk food by preparing mixtures of purified protein, lard,

<sup>7</sup> Osborne and Mendel: Feeding Experiments with Isolated Food-Substances, Carnegie Institution of Washington, Publication 156, Part II, 1911; The Rôle of Different Proteins in Nutrition and Growth, *Science*, xxxiv, pp. 722-732, 1911; The Rôle of Gliadin in Nutrition, this *Journal*, xii, pp. 473-510, 1912.

<sup>8</sup> Osborne and Mendel: Maintenance Experiments with Isolated Proteins, this *Journal*, xiii, pp. 233-276, 1912. Successful feeding trials extending over six months with similar diets on mice have been conducted by Dr. Wheeler; *Journ. of Exp. Zool.*, 1913 (in press).

<sup>9</sup> The food is in the form of a paste consisting of milk powder, 60 per cent; starch, 12 per cent; lard, 28 per cent (see p. 318).

starch and "protein-free milk." Such foods have been singularly efficient in promoting growth of young rats. Individual animals vary in respect to their capacity to grow on this food, a few stopping after sixty days of growth, others continuing to grow for one hundred days or more. After normal growth stops, the animals may remain at constant weight for a few days, or grow very slowly, and then suddenly decline and die unless a change is made in the diet. Both interesting features of these experiments, namely, the excellent earlier growth and the ultimate failure, are exemplified in charts II and III, where they may be compared with the almost invariable complete success that attends the use of the milk food. The conclusion seems inevitable, therefore, that the "protein-free milk foods" are deficient in, or completely lack, something which milk contains and which is indispensable for perfect growth.

This ultimate inhibition of growth, and nutritive decline in our feeding trials with the mixtures of isolated food stuffs is clearly connected with the diet factors. Our milk food has invariably brought prompt recovery and continuation of normal growth (see charts II and III). Even brief periods of milk feeding suffice to replenish, or provide, or permit to develop, that non-protein factor in the lack of which, cessation of growth ensues. If rats are allowed to grow on our "protein-free milk" food for some time, but are given milk food for a short period before growth ceases, or even after the decline has begun, the return to the "protein-free milk" food may again be attended with a long period of successful growth until there sets in a second inhibition, or decline, which can likewise be averted, or repaired by further exhibition of milk food.

All the essential factors for growth must be present in the diet if normal growth is to occur and continue. Failure to grow may result from a variety of factors some of which, like a deficiency of protein, or carbohydrate, or inorganic salts, or an inappropriate type or mixture of these nutrients, are apparent.<sup>10</sup> What light does the experience thus far accumulated throw upon the nature of the essential substance, if there be such? Is it organic or inorganic, or both?

<sup>10</sup> See further our discussion of the subject in *Zeitschr. f. physiol. Chem.*, lxxx, pp. 307-370, 1912.

We have already noted that ultimately failure invariably ensues when rats furnished a diet containing an adequate protein and "protein-free milk" have made a considerable part of their natural growth at a normal rate. Since no such failure to grow is observed when young rats are fed with the milk food, and since also those that have ceased to grow on the "protein-free milk" diet or have declined are promptly restored to satisfactory conditions of growth by the use of milk, it is evident that the latter contains something which our "protein-free milk foods" lack. It seems probable that the missing substance is organic in nature; for the "protein-free milk" may be presumed to contain all of the inorganic constituents of the milk. Nevertheless, in view of the limitations of our knowledge regarding minute quantities of elements which may play an important part in nutrition, hasty generalizations in this direction are scarcely permissible.

In a recent paper<sup>11</sup> we gave charts showing very considerable growth, at a normal rate, of the young white rat when supplied with a diet consisting solely of purified starch, lard, protein, lactose and inorganic salts; or, in other words, with foods containing our so-called "artificial protein-free milk," the preparation of which is described in the communication referred to. We also reported normal growth for a relatively long time on a similar diet in which the fat was replaced by carbohydrate. The number of these experiments, while not large, was sufficient to show that growth can be made on such diets; and the fact that they were conducted at different times and with several batches of food made with chemicals of different origin, excluded the possibility of error due to any accidental incorporation in the food of substances other than those they were intended to contain. These results were so different from any we had previously obtained with purely artificial diets<sup>12</sup> that we at once proceeded to confirm them by new experiments conducted on a much larger scale.

The chemicals used in making the first lot of "artificial protein-free milk" (designated I) were ordinary laboratory preparations

<sup>11</sup> Osborne and Mendel: Beobachtungen über Wachstum bei Fütterungsversuchen mit isolierten Nahrungssubstanzen, *Zeitschr. f. physiol. Chem.*, lxxx, pp. 307-370, 1912.

<sup>12</sup> Osborne and Mendel: Growth and Maintenance on Purely Artificial Diets, *Proc. Soc. for Exp. Biol. and Med.*, ix, p. 72, 1912; Beobachtungen über Wachstum bei Fütterungsversuchen mit isolierten Nahrungssubstanzen, *Zeitschr. f. physiol. Chem.*, lxxx, p. 356, 1912.

of good quality. Later we used Kahlbaum's preparations, and with the foods containing the "artificial protein-free milk" thus made (which we designate II) in many cases we obtained growth quite comparable with that previously secured with foods of similar character, but containing the natural "protein-free milk." The outcome of all our growth experiments with the "artificial protein-free milk" food mixtures I and II are shown in chart IV. For the new experiments special care was taken to use chemicals of a high degree of purity, as ascertained by careful analyses. To our surprise the "artificial protein-free milk," III, made with these purer chemicals failed in every case but one to promote more than slight growth (see chart V). Since the only apparent difference in the conditions under which these later trials were conducted was the greater purity of the chemicals used, our attention was at once turned to those inorganic elements which have been found in animal tissues in traces, but of the need of which in the diet nothing has as yet been learned. Traces of such elements may have been present as impurities in the chemicals first employed.

We accordingly made another preparation of "artificial protein-free milk," IV, to which traces of iodine, manganese, fluorine, and aluminium were added. The composition of these different preparations of "artificial protein-free milk" is shown by the table on following page.

Without a guide as to the proper amount of these "traces" of inorganic elements to add, the quantities in IV were chosen arbitrarily. These might, therefore, be either too much, or too little, in respect to any one or all of the additions.

An inspection of chart V shows plainly that much better growth was secured with preparation IV than with III. The findings may be summarized by saying that with "artificial protein-free milk" mixtures we have, under certain conditions, obtained a very considerable growth which, nevertheless, in most instances has ceased sooner than that induced by the natural "protein-free milk." The latter food, however, also invariably fails sooner or later to satisfy the nutritive requirement for growth.

McCollum and Davis,<sup>13</sup> whose significant experiments have con-

<sup>13</sup> McCollum, E. V. and Davis, M.: The Influence of the Composition and Amount of the Mineral Content of the Ration on Growth, *Proc. of Amer. Soc. of Biol. Chem.*, this *Journal*, xiv, p. xl, 1913.

• *Composition of "artificial protein-free milk."*

(Quantities used to make sufficient of the mixture to prepare 1 kgm. of food.)

	I, II,* III†	IV
	grams	grams
CaCO <sub>3</sub> .....	13.48	13.48
MgCO <sub>3</sub> .....	2.42	2.42
Na <sub>2</sub> CO <sub>3</sub> .....	14.04	3.42‡
K <sub>2</sub> CO <sub>3</sub> .....	14.13	14.13
H <sub>2</sub> PO <sub>4</sub> .....	10.32	10.32
HCl.....	12.75	5.34‡
H <sub>2</sub> SO <sub>4</sub> .....	0.92	0.92
Citric acid + H <sub>2</sub> O.....	10.10	11.11§
FeCl <sub>3</sub> ·1½H <sub>2</sub> O.....	0.634	0.634
KI.....		0.0020
MnSO <sub>4</sub> .....		0.0079
NaF.....		0.0062
K <sub>2</sub> Al <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub> .....		0.0024
Lactose.....	246.0	246.0

\* Prepared from Kahlbaum chemicals.

† Prepared from specially analyzed chemicals.

‡ The slight differences in the amounts of these compounds used in salt mixture IV, in contrast with I, II, and III, are due to the fact that in the latter, allowance was made for the sodium chloride produced by the neutralizing process in the preparation of our "protein-free milk." This addendum was omitted in IV in order to make its composition conform still more closely to that of the milk salts as such.

§ The small variations in the amounts of citric acid added are due to the fact that they were inadvertently made to correspond with two different analyses reported in the literature.

firmed ours in showing the possibilities of very considerable growth on the "artificial" dietaries, likewise appear to have encountered this cessation of growth for they state:

Rats grow normally during seventy-five to one hundred days on a ration consisting of pure casein, 18 per cent, dextrin, agar-agar and salt mixtures giving an inorganic content closely similar to either milk or egg yolk, and on certain other salt mixtures, in about the proportions found in milk and in eggs. With the same organic ration, fed with a salt mixture giving the ration an inorganic content closely similar to that of the wheat kernel, there is a complete suspension of growth. . . . Normal growth has been secured during seventy days on a ration of casein, 34 per cent, dextrin, agar-agar and a salt mixture giving an inorganic content similar in composition and quantity to that of dry skim milk.

The trenchant fact that failures can be averted or repaired by the use of milk foods leads to the inquiry wherein our "protein-

free milk" food differs from the conspicuously successful "milk food." The composition of three typical food mixtures is given for comparison.

	C		E		M
	per cent		per cent		per cent
Casein.....	18	Edestin.....	18	Milk powder*....	60
Starch.....	29	Starch.....	26	Starch.....	12
Lard.....	25	Lard.....	28	Lard.....	28
Protein-free milk	28	Protein-free milk.	28		

\* This product is the "Whole Milk Powder" supplied by the Merrell-Soule Company of Syracuse, N. Y. For the analysis see *Report of the Connecticut Agricultural Experiment Station, Food and Drug Products, 1909, p. 238.*

These contain, in every 100 grams:

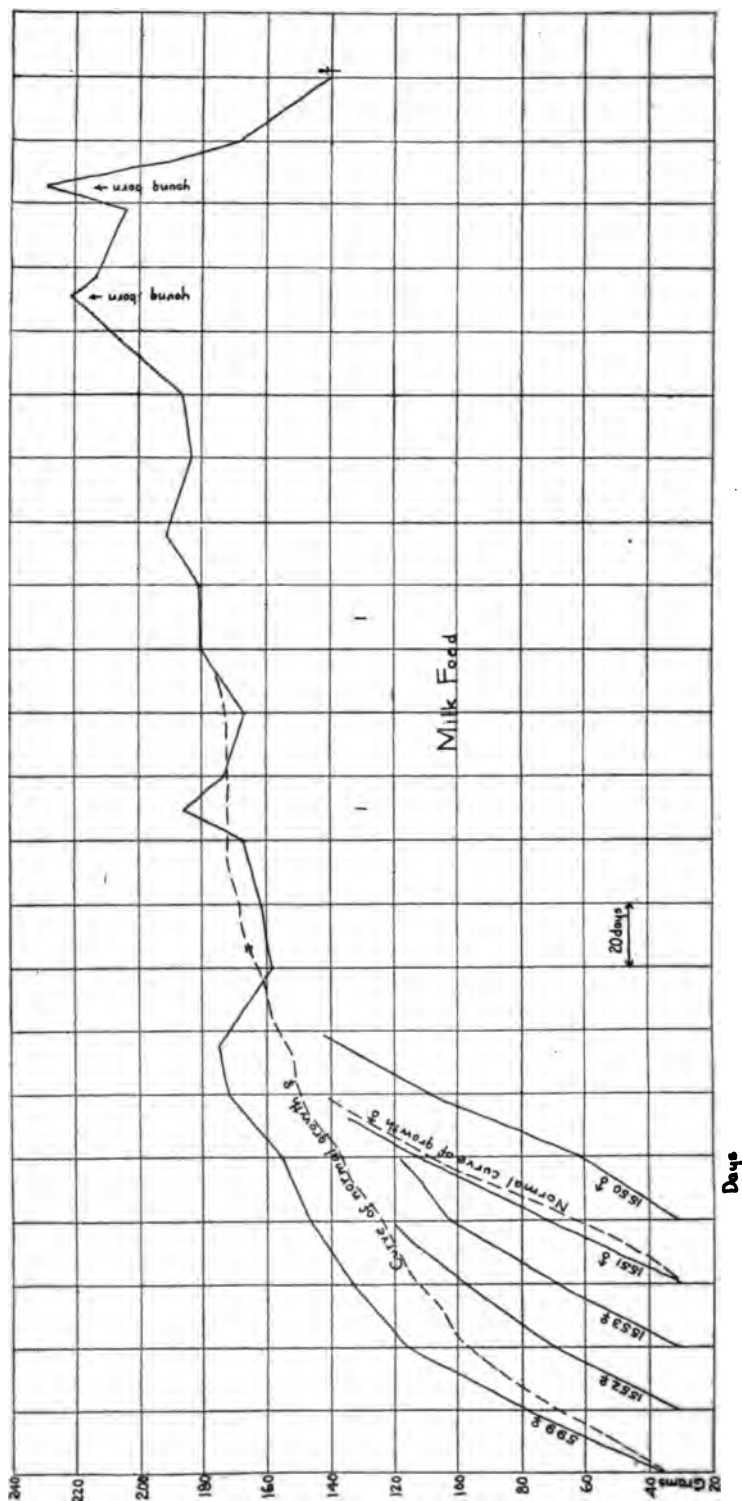
	C	E	M
	grams	grams	grams
Protein.....	18.0	18.0	15.4
Lactose.....	23.8	23.8	22.3
Starch.....	29.0	26.0	12.0
Milk salts.....	4.2	4.2	3.6
Total fats.....	25.0	28.0	44.4
Lard.....	25.0	28.0	28.0
Butter.....	0.0	0.0	16.4
Moisture.....	0.0	0.0	2.3

To what can we attribute the difference in the relative efficiency of the foods in promoting growth? First, not to the proteins, although these are unlike; for such evidence as we have already secured makes it extremely improbable that they are responsible for the nutritive differences. Second, not to the carbohydrate and inorganic constituents; for these are essentially alike in all the food mixtures. Third, not to the effect of the heat applied in the production of the "protein-free milk" component of the foods; for the milk powder used by us has also been subjected to an equally high temperature. An inspection of the tables discloses the fact that the foods C and E lack all those components of milk which are separated in the process of centrifugation of milk, *i.e.*, the cream and likewise any cellular elements



(mammary gland cells, leucocytes, bacteria, etc.) removed mechanically by the centrifugal process preliminary to the manufacture of our protein-free milk, or removed by subsequent filtration processes.

In seeking for the "essential" accessory factor we have, therefore, been led first of all to supply the cream component, in the form of butter, to rats which have ceased to grow on the "protein-free milk" foods. Numerous experiments still in progress have resulted in restoring rats, which have declined on the "protein-free milk" dietaries, to a weight normal for their age, quite as rapidly as does the efficient milk food. Examples of such recoveries are presented in charts VI and VII. Chart VI shows the effect of replacing part of the lard in our "natural protein-free milk" foods with a corresponding quantity of unsalted butter. It will be observed that after the cessation of growth, or after decline in body weight, recovery and renewed growth take place with the same rapidity as when the animals receive the milk food (compare charts II and III). Chart VII furnishes similar examples of recovery of animals which had ceased to grow on diets containing "artificial protein-free milk" IV, when part of the lard of this diet likewise was replaced with butter. These results are the more striking in view of the less rapid and continued growth manifested by most of the animals fed with the "artificial protein-free milk" foods. The illustrations presented in these charts are representative of a large number of similar experiments which we have conducted. It would seem, therefore, as if a substance exerting a marked influence upon growth were present in butter, and that this is largely, if not wholly, removed in the preparation of our natural "protein-free milk." Whether or not the latter is wholly deficient in this substance cannot be determined as yet from any data which we possess. It is true that young rats are able to make very considerable growth when fed on the natural "protein-free milk" diet; but possibly this is accomplished at the expense of some reserve substance stored in the cells of the young animal. It is too early to draw inferences as to the effective substance supplied by the butter. The detailed study of these important questions is being continued by us.



Days

CHART I. Typical curves showing normal growth of white rats on our milk food. Rat 599 ♀, after 371 days of growth and maintenance, gave birth to two litters of young. Rats 1550, 1551, 1552 and 1553 are the young of a mother fed on the milk food from the age of 59 days for 126 days prior to their birth. The normal growth of her young, which in turn were fed from the time of weaning on the milk food, is here represented. The ordinate represents grams of body weight, as indicated. The divisions of the abscissa represent 20-day periods.

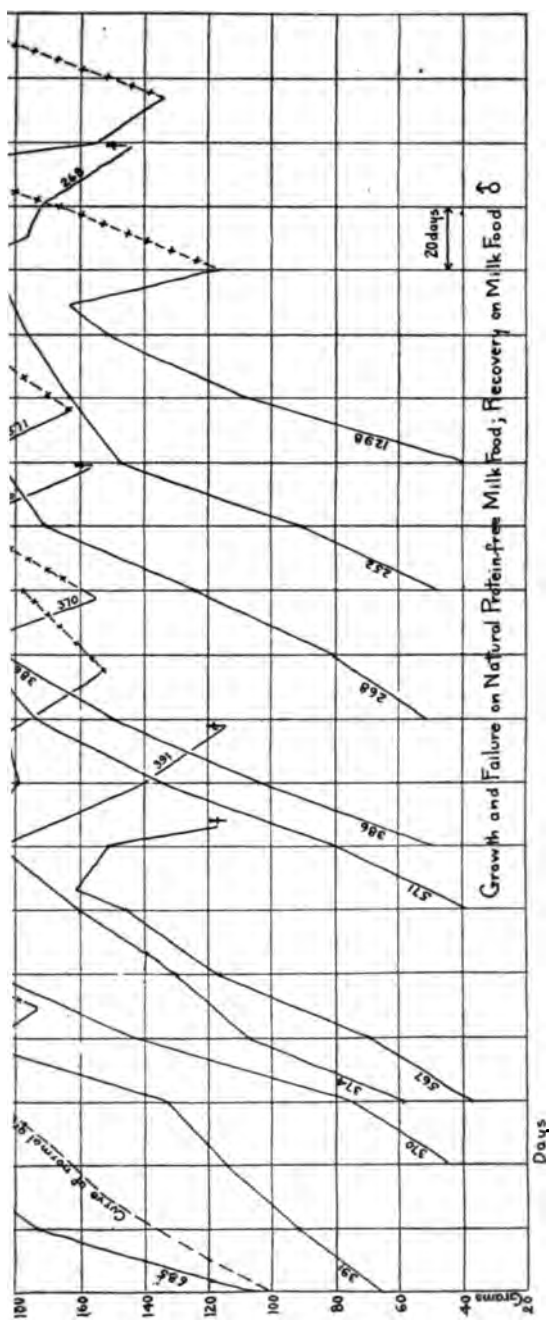


CHART II. Curves showing normal growth of *male* rats during many days, followed by cessation of growth, and decline on our "natural protein-free milk" foods. In every case where our milk food replaced the earlier mixture prompt recovery followed as indicated by the interrupted lines (x-x-x). The "protein-free milk" foods fed to the different rats contained various proteins as follows: casein, Rats 252, 268, 370, 386, 391; glutenin, Rat 374; lactalbumin, Rat 685; maize glutelin, Rat 567; ovovitellin, Rats 571, 1298.

The ordinates represent grams of body weight, as indicated. The divisions of the abscissa represent 20-day periods.

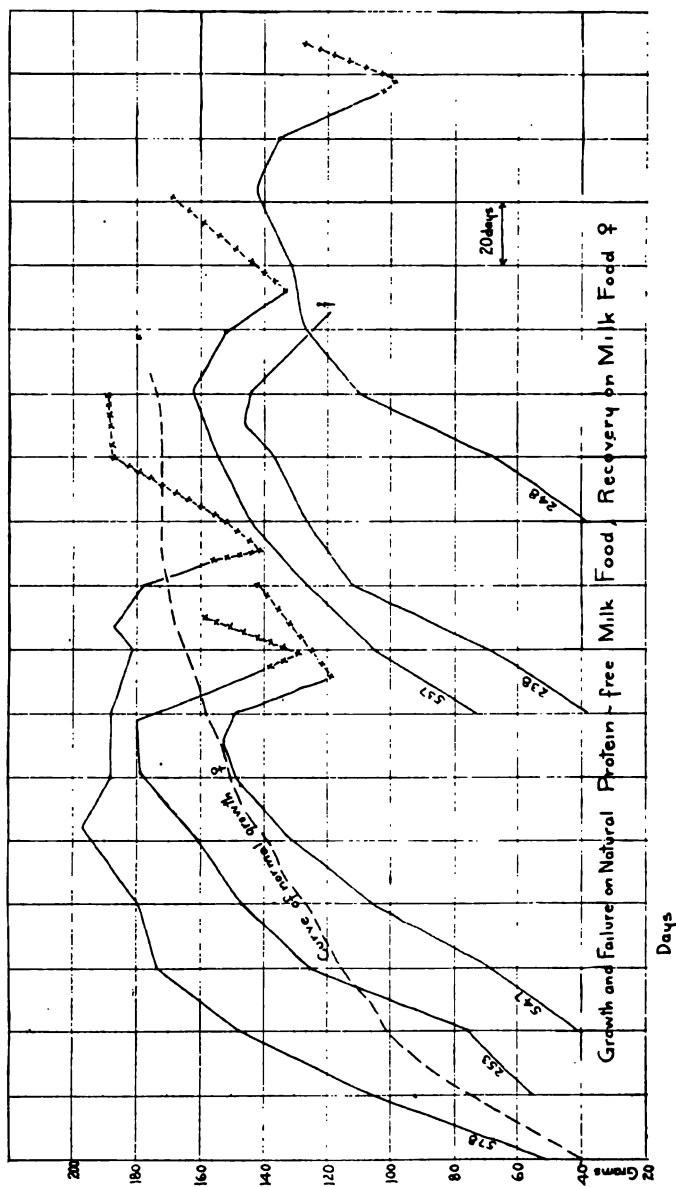


CHART III. Curves showing normal growth of female rats during many days, followed by cessation of growth, and decline on our natural protein-free milk foods. In every case where our milk food replaced the earlier mixture, prompt recovery followed as indicated by the interrupted lines (x-x-x-x). The "protein-free milk" foods fed to the different rats contained various proteins as follows: casein, Rat 238; edestin, Rats 248, 253; maize glutenin, Rat 547; ovovitellin, Rat 578; squash-seed globulin, Rat 537.

The ordinates represent grams of body weight. The abscissae represent 20-day periods.

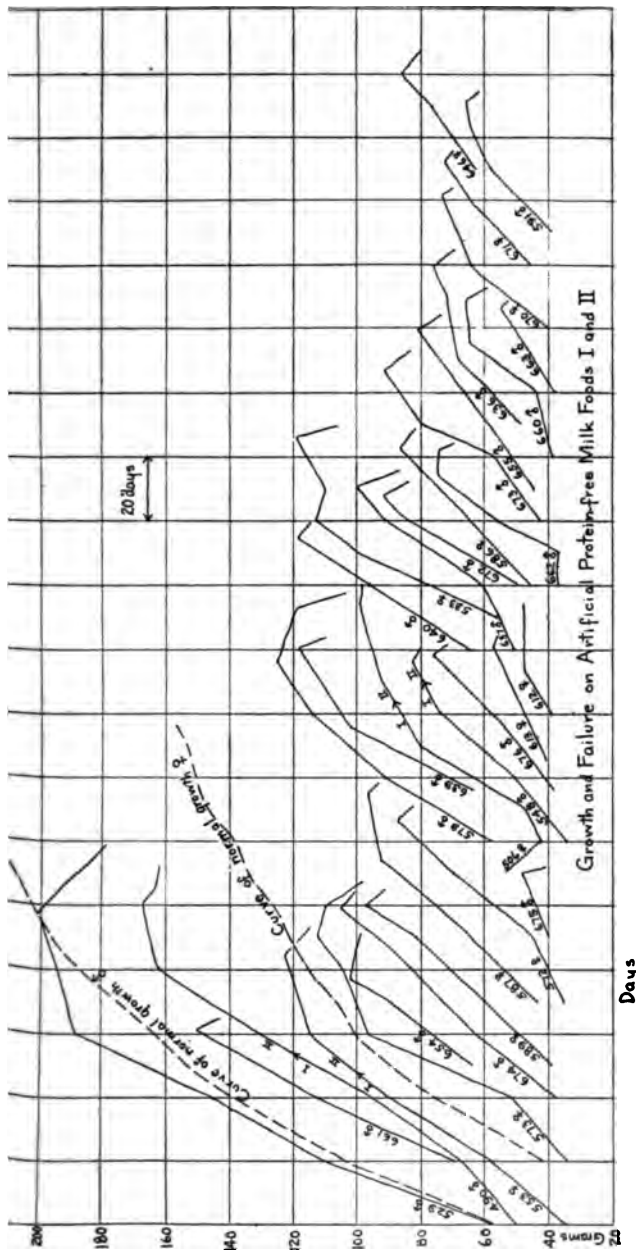


CHART IV. Curves of growth of all our rats fed on foods supplying the inorganic constituents in the form of "artificial protein-free milk," I and II. See page 317. Except where otherwise indicated in the chart, mixture II was used. It will be noted that in some cases growth was made at a rate comparable with that promoted by the "natural protein-free milk" as shown in charts II and III. In many cases the rats have more than doubled their weight. In general growth ceased sooner than when "natural protein-free milk" is fed.

The ordinates represent grams of body weight, as indicated. The divisions of the abscissa represent 20-day periods.

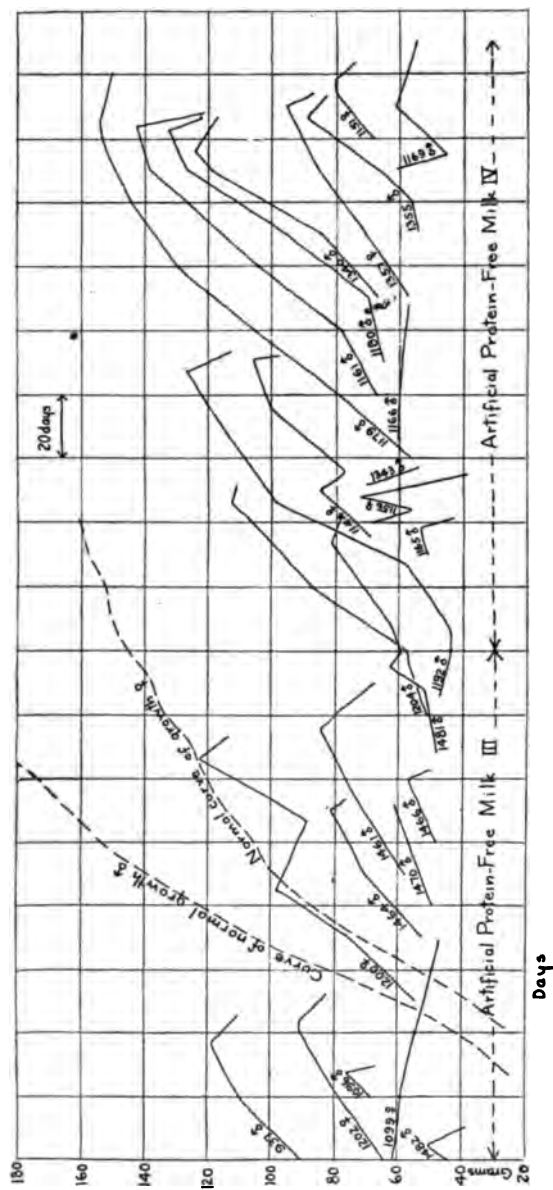


CHART V. Curves of growth of all our rats fed on foods supplying the inorganic constituents in the form of "artificial protein-free milk" III and IV. See page 317. Note the very slight growth made on III compared with that made on I, II (chart IV), and IV. The essential difference between III and IV is that the latter contained traces of Mn, Al, I and F.

The ordinates represent grams of body weight, as indicated. The divisions of the abscissa represent 20-day periods.

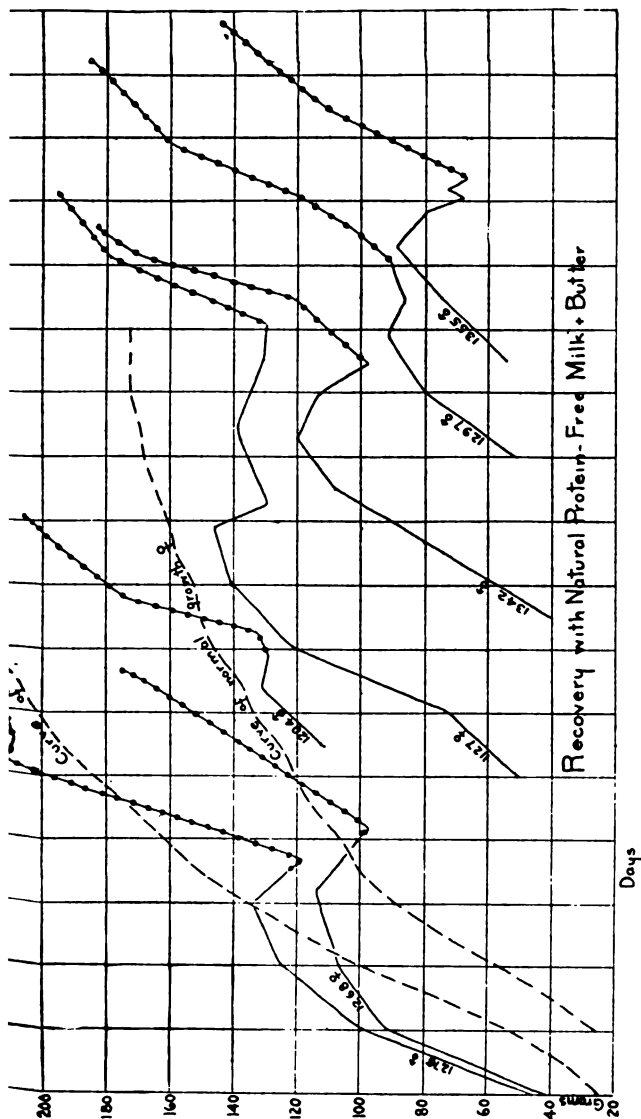


CHART VI. Curves of the body weight of rats which have ceased to grow or have declined on foods containing the natural "protein-free milk," and have recovered when part of the lard of the diet was replaced by a corresponding quantity of unsalted butter as indicated by the interrupted lines (o-o-o-o-o). The proteins furnished in the different experiments were as follows: casein, Rats 1204, 1268, 1276, 1297, 1342; edestin, Rats, 1127, 1355; ovalbumin, Rats 1268, 1276.

The ordinates represent grams of body weight, as indicated. The divisions of the abscissa represent 20-day periods.

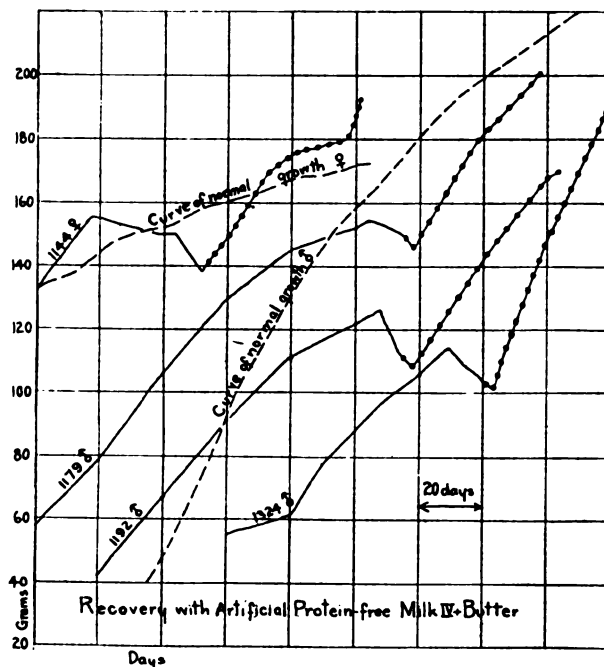


CHART VII. Curves of the body weight of rats which have ceased to grow or have declined on foods containing the "artificial protein-free milk" IV, and have recovered when part of the lard of the diet was replaced by a corresponding quantity of unsalted *butter* as indicated by the interrupted lines (o-o-o-o-o-o). The proteins furnished in the different experiments were as follows: casein, Rats 1144, 1192, 1324; edestin, Rat 1179; lactalbumin, Rat 1144.

The ordinates represent grams of body weight, as indicated. The divisions of the abscissa represent 20-day periods.



## STUDIES ON THE METABOLISM OF AMMONIUM SALTS.

### I. THE ELIMINATION OF INGESTED AMMONIUM SALTS IN THE DOG UPON AN ADEQUATE MIXED DIET.

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(Received for publication, June 23, 1913.)

The early recognition of the metabolic significance of ammonium salts has resulted in an extensive literature upon the behavior of these salts introduced into the organism. Upon the results obtained by Neubauer, Lohrer, v. Knierem, Coranda, Salkowski, Eder, Munk, Schmiedeberg and Walter, Hallervorden, Pohl and Junzer, Nencki, Salaskin, etc.,<sup>1</sup> rests for the most part our present conceptions regarding the behavior and function of ammonium salts in the animal body. In general the prime object of these experiments was the determination of the relation of ammonium salts to urea formation and the conclusions reached are too well known to need detailed repetition. It will be sufficient to state that all ammonium salts do not lead to urea formation in equal degree, thus those ammonium compounds that are the salts of organic acids may be completely transformed to urea, whereas ammonium salts of inorganic acids are only partially converted to urea, the remainder being eliminated as ammonium salts.

Apart from the relation of urea formation the influence of inorganic ammonium salts has received only scanty attention until recently. In the literature one may find a diversity of views as to the extent of urea formation from ammonium chloride, for example. This lack of uniformity has arisen from the fact that different investigators maintained unlike experimental conditions, such for example as diet, one employing animals receiving a mixed diet, another using dogs in a state of inanition. In

discussion of the problem, see Hammarsten: *Text Book of Physiology*, 1911.

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one instance in particular should attention be drawn to the experimental conditions existing. Feder<sup>2</sup> had shown that a major portion of ammonium chloride ingested by a dog reappeared in the urine as an ammonium salt. Munk<sup>3</sup> criticized Feder's results because fasting animals were employed. In his own investigation Munk used well-fed dogs only and in order to make the dog's urine resemble in alkalinity that of the rabbit (in which animal urea formation from inorganic ammonium salts is greater than in the dog) relatively large quantities of sodium acetate were given before and during the period of ammonium chloride administration. By this means he was able to demonstrate that as much as 53.55 per cent of the introduced ammonium salt reappeared in the urine as urea.

In view of the discordant opinions concerning the subject a systematic study has been made of the elimination of ammonium salts in dogs maintained upon a constant mixed diet. It was intended that the results obtained should serve as a basis for comparison of ammonia elimination under other experimental conditions of diet.

*Methods.* The observations were made upon two full-grown bitches accustomed to metabolism experiments. One of these animals was normal in every respect while from the other there had been removed,<sup>4</sup> several months previous to the periods of observation, a portion of the intestine for purposes bearing no relation to the present investigation. Both were fed upon a constant mixed diet consisting of fresh meat, cracker meal and lard, with a sufficiency of nitrogen and fuel value. The water intake was also constant. The ammonium salts were fed in gelatin capsules, the nitrogen content of which was too small to require consideration even though at times as many as six capsules were given per day. The ammonium salts, all Kahlbaum preparations, were analyzed for nitrogen and were then kept in glass stoppered bottles. The urine was divided into twenty-four-hour periods by catheterization, precautions being taken to prevent cystitis. Evidence of cystitis was never observed. Total nitrogen and ammo-

<sup>2</sup> Feder: *Zeitschr. f. Biol.*, xiii, p. 256, 1877.

<sup>3</sup> Munk: *Zeitschr. f. physiol. Chem.*, ii, p. 29, 1878-9.

<sup>4</sup> This animal was Dog A of previous experiments, cf. Underhill: *Amer. Journ. of Physiol.*, xxvii, p. 366, 1911.

nia nitrogen only were estimated. The urines obtained were always markedly acid to litmus.

From the data contained in Tables 1 and 2 it is apparent that when comparable quantities of nitrogen (approximately 1 gram) are orally introduced, in the form of ammonium salts of certain organic acids, into dogs maintained under constant dietary conditions none of the nitrogen reappears in the urine in the form of ammonium salts. This constitutes a direct confirmation of

TABLE 1.

Dog 1.

*Experiments with ammonium lactate and ammonium citrate.*

DATE 1910	BODY WEIGHT	URINE				REMARKS
		Volume	Specific gravity	Total N	Ammonia N	
	kilos	cc.		grams	gram	
March 11	6.9	200	1.020	4.23	0.17	The daily nitrogen intake in the food during this period amounted to 4.22 grams.
12	6.9	195	1.022	4.20	0.20	
13	6.9	180	1.020	4.29	0.19	
14	6.8	180	1.026	4.36	0.24	
15	6.9	200	1.030	5.24	0.22	{ 10.41 grams ammonium lactate = 1.07 grams N were fed in 5 equal portions.
16	6.9	130	1.040	4.37	0.19	
17	6.9	180	1.020	4.27	0.19	
18	6.8	175	1.035	5.64	0.22	{ 9.07 grams of ammonium citrate = 1.10 grams N were fed in 5 equal portions.
19	6.9	220	1.035	4.25	0.20	
20	6.9	125	1.040	4.14	0.23	

some of the older investigations. That the absorption of these salts was complete is attested by the output of total nitrogen. It may be inferred that under the experimental conditions ammonium salts ingested in the form of the lactate, acetate, butyrate and valerianate are completely transformed to urea. Moreover, in spite of the fact that a fairly large portion of the small intestine had been removed from Dog 2 no evidence of any difference from the normal animal could be detected in the reaction under discussion. In neither animal were abnormal symptoms observed

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after ingestion of any of the ammonium salts with the exception of the valerianate which invariably induced nausea and vomiting. After several trials with this salt a single clear-cut result was finally obtained, Table 2, Dog 2. As may be seen from Table 3

TABLE 2.

*Dog 2.*

*Experiments with ammonium acetate, ammonium butyrate and ammonium valerianate.*

DATE 1910	BODY WEIGHT	URINE				REMARKS
		Volume	Specific gravity	Total N	Ammonia N	
March	kilos	cc.		grams	gram	
6	8.9	200	1.020	3.96	0.25	During this period the daily intake of food N amounted to 5.29 grams.
7	9.0	140	1.035	3.90	0.27	
8	9.0	155	1.026	3.75	0.25	
9	9.0	250	1.017	3.84	0.25	
10	9.0	200	1.020	4.98	0.24	{ 6.65 grams ammonium acetate = 1.08 grams N were fed in 4 equal portions.
11	9.0	180	1.020	3.99	0.24	
12	9.0	175	1.025	3.75	0.22	
13	9.0	140	1.030	3.95	0.25	
14	8.9	195	1.026	5.16	0.23	{ 10.25 grams ammonium butyrate = 1.09 grams N were fed in 6 equal portions.
15	9.0	120	1.024	3.75	0.25	
16	8.9	150	1.030	3.72	0.25	
17	8.9	140	1.035	3.75	0.23	
18	8.9	240	1.035	4.80	0.24	{ 13.36 grams ammonium valerianate = 1.08 grams N were fed in 5 equal portions.
19	8.9	125	1.036	3.78	0.25	
20	8.9	110	1.040	3.81	0.26	

ammonium carbonate behaves in manner identical with the other ammonium salts just discussed.

When ammonium salts of the inorganic acids are considered, entirely different conclusions must be drawn—see Tables 3, 4 and 5. With ammonium chloride ingestion ammonia nitrogen vary-

ing from 41 per cent of that ingested (with Dog 2, Table 5) to 47 per cent and 52 per cent (with Dog 1, Table 3) was eliminated in the urine, the period of excretion being complete within 48 hours. With Dog 2 equal quantities of the excess of ammonium

TABLE 3.

Dog 1.

*Experiments with ammonium carbonate and ammonium chloride.*

DATE 1910	BODY WEIGHT	URINE				REMARKS
		Volume	Specific gravity	Total N	Ammonia N	
February	<i>lbs</i>	<i>cc.</i>		<i>grams</i>	<i>gram</i>	
21	7.0	150	1.025	3.75	0.20	Throughout this period the daily diet contained 4.16 grams N.
22	7.0	160	1.028	3.85	0.26	
23	7.0	130	1.030	3.79	0.28	
24	7.1	275	1.017	3.87	0.23	
25	7.0	150	1.032	3.85	0.21	
26	7.0	150	1.033	5.19	0.26	{ 5.52 grams ammonium carbonate containing 0.95 gram N were fed, divided into 3 portions.
27	7.0	225	1.017	3.95	0.23	
28	7.0	220	1.032	6.00	0.57	{ 3.94 grams ammonium chloride = 1.02 grams N fed in 3 portions.
March 1	7.0	220	1.030	5.01	0.38	
19	6.9	220	1.035	4.25	0.20	The daily nitrogen intake in food during this period amounted to 4.22 grams.  { 3.96 grams of ammonium chloride = 1.02 grams N were fed in 4 equal portions.
20	6.9	125	1.040	4.14	0.23	
21	6.9	300	1.020	5.37	0.57	
22	6.8	140	1.035	3.98	0.42	
23	6.8	100	1.040	3.76	0.23	

salt were eliminated on each of the two days of excretion. In the first experiments with Dog 1 (February 28, Table 3) 36 per cent reappeared on the first day and 11 per cent on the second. The second experiment (March 21, Table 3) demonstrated that

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36 per cent of the ammonium salt ingested reappeared in the urine during the first day and 16 per cent on the second day.

After the introduction of ammonium phosphate (Table 4, Dog 1) there appeared in the urine as ammonia nitrogen about 64 per cent of that ingested. On the first day 28 per cent was eliminated, on the second 26 per cent and on the third day 10 per cent. Of the inorganic ammonium salts the nitrogen of the sul-

TABLE 4.

*Dog 1.*

*Experiments with ammonium phosphate and sodium chloride.*

DATE 1910	BODY WEIGHT	URINE				REMARKS
		Volume	Specific gravity	Total N	Ammonia N	
	kilos	cc.		grams	gram	
April 8	7.0	200	1.025	4.14	0.20	The nitrogen ingested in the food daily amounted to 4.22 grams.
9	7.0	210	1.023	4.02	0.21	
10	7.0	220	1.020	4.08	0.19	
11	7.0	175	1.030	4.00	0.19	
12	7.0	225	1.048	5.46	0.51	{ 9.6 grams ammonium phosphate = 1.11 grams N were fed in 5 equal portions.
13	6.9	125	1.035	4.14	0.49	
14	6.9	125	1.030	3.60	0.32	
15	7.0	110	1.032	3.58	0.18	
16	7.0	180	1.025	3.81	0.18	{ 4.35 grams sodium chloride fed in 3 equal portions.
17	7.0	170	1.028	3.76	0.17	
18	7.0	240	0.021	3.64	0.06	
19	6.9	80	1.038	3.46	0.17	
20	7.0	100	1.034	3.65	0.18	

phate (Table 5) seems to be more completely converted into urea than any of the others tested since only 29 per cent of the nitrogen introduced as ammonium salt was eliminated in the urine as ammonia, 18 per cent appearing on the first day and 11 per cent on the second day. With all of the inorganic ammonium salts there may be noticed a slight excess of total nitrogen excreted over the average output. In general this excess of nitrogen in

the urine is observable only on the day of ingestion although with the introduction of ammonium chloride on February 28 (Table 3) the augmented total nitrogen excretion persisted during

TABLE 5.

*Dog 2.*

*Experiments with ammonium chloride, ammonium sulphate and sodium chloride.*

DATE 1910	BODY WEIGHT	URINE				REMARKS
		Volume	Specific gravity	Total N	Ammonia N	
<b>March</b>	<i>kiloe</i>	<i>cc.</i>		<i>grams</i>	<i>gram</i>	
19	8.9	125	1.036	3.78	0.25	The daily intake of food N amounted to 5.29 grams. { 4.21 grams ammonium chloride = 1.08 grams N were fed in 4 equal portions.
20	8.9	110	1.040	3.81	0.26	
21	9.0	325	1.019	5.01	0.49	
22	8.9	110	1.040	3.81	0.46	
23	8.9	175	1.030	3.90	0.26	
<b>April</b>						
8	9.8	150	1.030	3.80	0.26	{ 5.01 grams ammonium sulphate = 1.03 grams N were fed in 3 equal portions.
9	9.8	145	1.032	3.87	0.25	
10	9.8	120	1.036	3.92	0.22	
11	9.8	120	1.038	3.96	0.23	
12	9.8	200	1.042	5.13	0.44	
13	9.7	125	1.030	3.89	0.37	{ 4.51 grams sodium chloride were fed in 3 equal portions.
14	9.7	100	1.035	3.87	0.27	
15	9.7	110	1.032	3.90	0.21	
16	9.8	110	1.032	3.81	0.23	
17	9.8	100	1.036	3.69	0.23	
18	9.8	240	1.020	3.91	0.12	
19	9.8	90	1.036	3.78	0.26	
20	9.8	100	1.035	3.81	0.24	

**March 1.** It is apparent that the ingestion of ammonium salts of inorganic acids usually causes an output of total nitrogen in distinct excess of the usual elimination previous to the introduction of the ammonium salt. During this period a portion of the

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ingested ammonia nitrogen is retained within the organism and the query arises whether the retention of the ammonium compound is the cause of the greater output of total nitrogen. Does the ammonium salt bring about a temporary stimulation of nitrogenous catabolism? To obtain an answer to this question whether the retention of ammonium salts is responsible for the excess of nitrogen eliminated one would turn naturally to the results obtained with the introduction of ammonium salts of the organic acids since in no instance was there retention of ammonia nitrogen. Upon inspection it is seen that the results obtained here are conflicting with respect to the subject under discussion for with the citrate and butyrate excess of total nitrogen comparable with that seen with inorganic ammonium salts may be noticed. With the acetate and valerianate no change is noticeable while with the lactate the output of total nitrogen is slightly under the normal average excretion. It is probable therefore that the more prolonged retention of inorganic ammonium salts cannot be the fundamental reason for the excess of total nitrogen eliminated. On the other hand, one may conclude that some ammonium salts, whether of organic or inorganic nature, possess the property of causing the output of a small excess of total nitrogen, which may perhaps be regarded as a slight stimulation to nitrogenous catabolism.

No adequate explanation exists for the temporary retention of the ammonium salts of inorganic acids unless indeed it is assumed that these compounds are more or less toxic and therefore must be temporarily stored until the organism can eliminate them without injury to itself. It may be accepted probably that the acid radicle is responsible for this behavior and it is possible that excess of the acid in the cells requires ammonia for its storage and subsequent excretion. To determine whether an inorganic acid ingested as a neutral salt is capable of exerting any influence upon the output of ammonia nitrogen an amount of chloride in the form of sodium chloride equivalent to that ingested as ammonium chloride was introduced under the usual conditions—see Tables 4 and 5. It will be observed from these data that no influence was exerted by sodium chloride upon the total nitrogen output nor was the ammonia nitrogen elimination increased. Instead, in both instances the ammonia nitrogen excretion was diminished to one-half the usual amount.



## SUMMARY.

Ammonium salts of a number of organic acids ingested by dogs maintained upon a constant mixed diet failed to increase the ammonia nitrogen output in the urine.

Introduced under comparable conditions ammonium salts of some of the inorganic acids caused a varying degree of increase in the urinary ammonia nitrogen. The experiments afford no adequate explanation for this temporary retention of the ammonium salts.

All the *inorganic* ammonium salts tested and some of those of *organic* nature cause a distinct excess of total nitrogen output over the normal. These salts apparently stimulate nitrogenous catabolism.

Sodium chloride fed under the experimental conditions causes a distinct lowering of the ammonia nitrogen elimination.



## STUDIES ON THE METABOLISM OF AMMONIUM SALTS.

### II. A NOTE ON THE ELIMINATION OF INGESTED AMMONIUM SALTS DURING A PERIOD OF PROLONGED INANITION.

By FRANK P. UNDERHILL.

*(From the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven, Connecticut.)*

(Received for publication, June 23, 1913.)

The object of the present investigation is to determine whether a dog in a poor nutritive condition especially with respect to carbohydrate store, behaves differently than a well-fed animal when ammonium salts are ingested. In order to make the body as free as possible from glycogen phlorhizin was administered, in exact accordance with the method of Lusk, for a period of three days, beginning January 19 (see table), the urine for the last two days only being collected. Upon these days a condition of total diabetes was attained. From January 19 forward no food was given, otherwise the experimental conditions existing were in every way comparable with those of the preceding paper.

It will be noted from the figures given in the table that in spite of the probable comparative paucity of glycogen the urinary nitrogenous constituents determined failed to show any definite changed relationships. The failure of any change in this respect tends to demonstrate that during prolonged starvation when the carbohydrate store is at a minimum the organism of the dog still retains the ability to eliminate ammonia arising within the body in a normal manner, namely, as urea. Moreover, when ammonium carbonate is ingested the subsequent ammonia nitrogen excretion is in every respect similar to that of the normal well-fed animal.

The ingestion of ammonium chloride was followed by a period during which the ammonia nitrogen elimination rose far above the normal and was maintained at a high level throughout the remainder of the experiment. The reason for this is problemati-

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cal. The fact that the ammonia nitrogen rose from 0.15 gram to 0.36 gram may at least be accepted as indicating that some of the ammonium salt was temporarily retained in a manner similar to that seen with the well-fed dog. With the first ingestion of ammonium carbonate little or no excessive amount of total nitrogen output is to be observed. When ammonium chloride was introduced, however, a marked rise in total nitrogen was in evidence and the former level failed to be regained. The second ingestion of ammonium carbonate also was followed by a marked augmentation of total nitrogen excretion which did not regain the former level. It appears hardly possible that these relatively large increases in total nitrogen output could have been merely coincident with the intake of the ammonium salts without bearing some definite relation to them. It seems more reasonable to assume that the ammonium salts exert a much greater action in this direction during a period when presumably the organism must be in a condition of unusual susceptibility, than is possible under normal circumstances.

#### CONCLUSIONS.

From the data presented it is indicated that during a period of prolonged inanition the ingestion of ammonium carbonate by the dog fails to show any increase in the urinary ammonia-nitrogen output.

Ammonium chloride, on the other hand, causes a marked increase in ammonia nitrogen which subsequently remains at a high level. The salt is responsible also for a noticeable increase in the total nitrogen output which fails to regain the former level. At this stage a second ingestion of ammonium carbonate may also bring about a significant augmentation of the total nitrogen elimination.

*The ingestion of ammonium salts during prolonged inanition.*

DATE 1910	BODY WEIGHT	URINE				REMARKS
		Volume	Specific Gravity	Total N	Ammonia N	
	kilos	cc.		grams	grams	
<b>January</b>						
20	12.7	475	1.060	12.72	0.74	D : N ratio = 3.77.
21	12.5	450	1.064	8.94	0.50	D : N ratio = 3.47.
22	12.3	925	1.035	11.02	0.55	
23	11.7	600	1.035	8.64	0.64	
24	11.1	450	1.033	7.92	0.45	
25	10.9	420	1.030	5.10	0.20	
26	10.6	260	1.020	3.60	0.09	
27	10.3	110	1.030	2.85	0.18	
28	10.2	190	1.020	2.91	0.19	
29	10.0	Urine contaminated.				
30	9.8	Urine contaminated.				
31	9.5	180	1.019	3.09	0.20	
<b>February</b>						
1	9.2	90	1.025	2.34	0.13	
2	9.0	100	1.030	2.40	0.16	{ 5.39 grams ammonium carbonate = 0.93 gram N were fed in 3 equal portions.
3	9.0	120	1.030	3.42	0.15	
4	8.9	60	1.045	2.26	0.11	
5	8.9	100	1.035	2.31	0.15	{ 3.94 grams ammonium chloride = 1.01 gram N were fed in 3 equal portions.
6	8.8	100	1.040	4.20	0.36	
7	8.6	100	1.025	3.15	0.36	
8	8.1	90	1.035	3.45	0.53	
9	8.1	100	1.030	3.96	0.34	{ 5.53 grams ammonium carbonate = 0.95 gram N were fed in 3 equal portions.
10	7.9	130	1.035	5.32	0.36	
11	7.9	120	1.040	4.96	0.32	
12	7.7	150	1.035	6.30	0.30	
						The urine was always strongly acid to litmus.



## STUDIES ON THE METABOLISM OF AMMONIUM SALTS.

### THE UTILIZATION OF AMMONIUM SALTS WITH A NON-NITROGENOUS DIET.

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(Received for publication, June 23, 1913.)

The possible rôle of ammonium salts in intermediary metabolism has been emphasized by the recent communication of Grafe and Schläpfer,<sup>1</sup> according to whom there is a notable retention of nitrogen when these salts are ingested by dogs maintained on diets rich in carbohydrate and fat but containing little protein. It is the opinion of these investigators that under such circumstances the retained nitrogen is combined with carbohydrate to form an amino-acid complex, hence protein synthesis occurs. Abderhalden<sup>2</sup> has confirmed the general results obtained

Grafe and Schläpfer but denies the probability of a protein synthesis with ingested ammonium salts as the source of nitrogen. Grafe and Schläpfer insist that very large quantities of carbohydrates are essential in order to obtain a satisfactory demonstration of ammonia utilization and Abderhalden has employed accordingly diets of large calorific value. On the other hand Taylor and Ringer<sup>3</sup> have shown that "the presence of carbohydrates is not an obligatory factor in the retention of nitrogen on ammonia."

The observations of Grafe and Schläpfer lead to the inference that the character of the ammonium salts employed is a matter of indifference, ammonium salts of inorganic acids, as ammonium chloride, or of organic acids, as the citrate, showing the same

Grafe and Schläpfer: *Zeitschr. f. physiol. Chem.*, lxxvii, p. 1, 1912.

Abderhalden: *Ibid.*, lxxviii, p. 1, 1912, and lxxxi, p. 1, 1912. See also check: *Biochem. Zeitschr.*, xlv, p. 244, 1912.

Taylor and Ringer: *this Journal*, xiv, p. 407, 1913.

type of action—varying only in degree. In view of the radically different mode of elimination of the two types of ammonium salts in dogs during starvation and also upon an adequate mixed diet one might naturally expect to find a marked difference in the character of the influence exerted upon intermediary metabolism under the experimental conditions here discussed. The determination of the urinary ammonia nitrogen output under these circumstances should contribute toward the solution of the problem. Such estimations, fundamental for the question at hand, have been made neither by Grafe and Schläpfer nor by Abderhalden.

A review of the work of Grafe and Schläpfer makes it evident that criticism may be directed at the methods of procedure adopted. In their recital of the technique employed it is stated

Vom täglichen Katheterisieren wurde abgesehen, da selbst, wenn die hintere Scheidenwand nicht gespalten wird, die Gefahr einer Cystitis, die unter allen Umständen vermeiden werden musste, bei den jungen Tieren zu gross ist. Da Periode nur mit Periode, nicht Tag mit Tag verglichen wurde, konnte ohne Beeinträchtigung der Genauigkeit der Versuche der tägliche Katheterismus unterlassen werden.<sup>4</sup>

For the sake of clarity a portion of the protocols of Grafe and Schläpfer are reproduced on the following page.

In view of the last statement quoted above one may query why the average of the last three days of the second fore-period should be employed as a standard representative of this period. In the absence of catheterization what is the evidence that a goodly portion of the urine properly belonging to the fifteenth experimental day was not voided on the sixteenth day? The adoption of such a procedure in metabolism investigations is always unjustifiable, and it is especially so in this instance where the point of the entire study rests upon the strictest accuracy. In the partial reproduction of "Versuch II" one may look askance at the results with even more reason, for here the nitrogen elimination of the second fore-period was extremely variable. What justification is there for selecting the average of the last two days as a standard? Again, in the experimental period (not reproduced) a whole day, the eighteenth, is excluded because a por-

<sup>4</sup> Grafe and Schläpfer: *loc. cit.*, p. 4.



Versuch I. Fox, männlich (Zulage von 0,5 g N als  $\text{NH}_4\text{Cl}$ ).

N-Gehalt der Nahrung g	Urinmenge ccm	N-Gehalt des Urins g	N-Gehalt des Kotes g	N-Bilanz pro die	N-Retention verglichen mit dem Mittelwert von Vor- und Nachperiode	Bemerkungen
0,0725	360 incl. Spül- wasser	1,86	—	—	—	Vorperiode II.
0,0725	225	0,971	0,048	-0,946	Bei einer	
0,0725	125	0,838	0,048	-0,813	N-Einfuhr von	
0,0725	210	0,802	0,048	-0,777	0,0725 g N pro	
0,0725	225	0,980	0,048	-0,954	die wurden in	
0,0725	?	?	0,048	?	den letzten 3	An beiden Tagen
0,0725	?	?	0,048	?	Tagen im	etwas Urin verlo-
0,0725	580	0,741	0,048	-0,716	Durchschnitt	ren gegangen.
					0,849 g N	
0,0725	460	0,811	0,048	-0,786	pro die	
0,0725	350	0,85	0,048	-0,825	abgegeben	
0,0725	480	1,23	0,036	-0,753	Bei einer täg-	Hauptperiode.
0,0725	480	1,119	0,036	-0,7319	lichen Zer-	
0,0725	380	0,752	0,036	-0,275	setzung von	
0,0725	355	1,001	0,036	-0,524	0,8627 g N und	0,5 g N in Form
					einer Einfuhr	von $\text{NH}_4\text{Cl}$ pro die.
					von 0,5 g täglich	
					werden pro die	
					1,121 g N aus-	
					geschlossen,	
					d. h. 0,242 g N	
					retiniert = 50%	
					der Einfuhr	
0,0725	450	0,851	0,050	-0,828	Bei 0,0725 g	Nachperiode.
0,0725	320	0,84	0,050	-0,817	N-Einfuhr	
0,0725	370	0,821	0,050	-0,898	verliert der	
0,0725	—	—	—	—	Körper pro die	
					0,8763 g N	

Versuch II. Hund, Ami ♀ (Zulage von 1,0 g N als  $\text{NH}_4\text{Cl}$ ).

0,1016	104	2,735	0,041	—	In den letzten	Vorperiode II.
					Tagen N-Ver-	
0,1016	450	1,268	0,041	-1,207	lust des Kör-	Etwas Durchfall.
0,1016	550	0,963	0,041	-0,902	pers pro die	" "
0,1010	200	0,546	0,041	-0,485	= 0,796 bei	(20 Tr. Tinct. opii)
					Aufnahme	" "
					von 0,1016 g	
					pro die	

tion of the urine was lost, but even making this allowance the end result is probably far from correct.

Although the adoption of the method in this case would alter very little the result one is impelled to protest against the practice of taking the average of the fore- and after-periods as a measure of comparison for the experimental period. In this way any possible influence extending beyond the experimental period is overlooked.

Turning again to the description of the technique of Grafe and Schläpfer<sup>5</sup> we read

Der Stuhl wurde getrennt aufgefangen und gesammelt. Da die Tiere besonders bei den Versuchen mit Chlorammonium hin und wieder Durchfall bekamen, war eine Trennung natürlich nicht immer möglich. Es wurde dann der Stuhl durch Filtration des Urins und des Spülwassers gewonnen. Bei diesem wohl nicht zu umgehenden Verfahren konnte natürlich nicht verhindert werden, dass ein Teil des Kotstickstoffs als Urinstickstoff gerechnet wird. Die Mengen, um die es sich dabei handeln kann, sind allerdings sehr klein. Hin und wieder besonders gegen Ende der Hauptperiode erbrachen die Tiere etwas. Auch hier ist natürlich eine Trennung des Erbrochenen von Stuhl und Urin oft nicht möglich. Die festeren Bestandteile der Nahrung blieben natürlich mit dem Kot, wenn solcher nicht getrennt gesammelt werden konnte, auf dem Filter zurück, während das Ammoniumsalz mit ins Filtrat ging. Wie die ausserordentlich geringe Menge des Trockenkots (Filtrerrückstand) beweist, handelte es sich, wenn überhaupt, stets um sehr kleine Menge erbrochener Nahrung. An den Tagen, an welchen Durchfall und Erbrechen vorhanden war (vgl. die Tabellen), umschliesst die N-Bestimmung im Harn also auch einen grossen Teil des nicht resorbierten Stickstoffs; die N-Werte im Urin stellen dann also Maximalzahlen dar.

It is evident from their own description that Grafe and Schläpfer recognize that their work makes little or no pretense to accuracy. Why then in view of the many acknowledged opportunities for relatively great errors should so much emphasis be laid upon the apparent retention of small quantities of nitrogen?

If the tables dealing with the other ammonium salts, ammonium acetate and citrate, are examined less criticism is warranted although here also at times the same type of error is introduced. For example, in Versuch III, in the experimental period the nitrogen for three days is questioned, and apparently the authors

<sup>5</sup> Grafe and Schläpfer: *loc. cit.*, p. 4.

consider that it is only necessary to exclude these days in order to have their results assume a condition of accuracy. In the very last period of the experiment why should the nitrogen of the feces be accepted since the figures given were obtained for the second fore-period twenty-five days previously? The conclusions derived from experiments with such possibilities for error must be regarded with a certain degree of skepticism. This, in relation to the significance of the theory advanced, was the impetus for the investigation detailed below, which in a general way is a repetition of the experiments of Grafe and Schläpfer, attempts being made to eliminate all possible sources of error.

*Methods.* The general plan of experimentation employed by Grafe and Schläpfer was adopted. For several days previous to the actual period of the investigation the animals were allowed to fast. This period was succeeded by one in which a non-nitrogenous diet was given and the real fore-period was begun only when the urinary nitrogen excretion had attained a more or less constant level. The feces of each period were marked off by administration of carmine, and catheterization divided the urine into daily periods. No indication of cystitis was ever observed. The diet consisted of a fine grade of cornstarch, refined lard, sucrose, bone ash and a constant quantity of water. The individual foodstuffs contained no measurable quantity of nitrogen. The animals were fed twice daily, at 8.40 a.m. and 5 p.m., during the fore- and after-periods. Throughout the experimental period the food and the ammonium salt were fed in three portions, the extra period being at noon. The food was prepared fresh for each meal and was treated as follows: The starch was made into a thick paste, the sugar, lard and bone ash added and the whole thoroughly mixed. As a rule the food was fed while warm. The ammonium salt was placed within a portion of the lard and this was first fed to the dog from a spoon, the remainder of the ration usually being greedily devoured. Toward the end of the experiments the animals at times showed a marked disinclination to eat and under these circumstances it became necessary to administer the food with a spoon. This was particularly true for Dog 2, whereas with Dog 1 such a procedure was unnecessary throughout the first experiment. Diarrhoea was never in evidence. The ammonium salts were always analyzed for nitrogen just previous to the time of administration and they were then kept in glass stoppered bottles. Chloride was estimated according to the Volhard procedure, trials having demonstrated that the results obtained in this way were identical with those derived by use of the Grüber<sup>6</sup> method. The determination of the other substances was carried through by the well-known methods and calls for no further comment. The urine was acid to litmus throughout all the periods.

<sup>6</sup> Grüber: *Zeitschr. f. Biol.*, xix, p. 569.

*Experiments with ammonium chloride.*

In previous experiments it has been demonstrated that ammonium chloride shows a decided difference from organic ammonium salts in its mode of elimination from the body during inanition or with an adequate mixed diet. Under these circumstances it seemed especially desirable to repeat the work of Grafe and Schläpfer with ammonium chloride to determine whether the nitrogen of this salt, in the absence of food nitrogen, would show a behavior with respect to its manner of elimination at all comparable to what had been observed under the previous experimental dietary conditions. Therefore, in addition to the determination of nitrogen balances, ammonia nitrogen excretion in the urine has been followed with the idea that this estimation might indicate better the type of processes taking place than could be derived from total nitrogen determinations only. In order to follow intermediary processes even more exactly daily chloride estimations were also made. With a similar object in view the elimination of creatinine and creatine was followed.

In Tables 1 and 2 are recorded the results obtained from feeding ammonium chloride to dogs kept upon an otherwise nitrogen-free diet but with relatively high fuel value. The contention of Grafe and Schläpfer that the nitrogen of ammonium chloride may diminish the negative nitrogen balance under the experimental conditions is in nowise corroborated by the data here presented. Comparing the average balance of the fore-period with that of the experimental period little or no difference can be found. Judged by the criterion of Grafe and Schläpfer therefore all of the nitrogen of the ammonium salt would appear to have been promptly eliminated, contrary to what they have maintained for their own experiments. A closer inspection of our ammonia figures, however, will show that not all of the ammonium salt has been so promptly excreted. There is apparently a lag in the elimination of the ingested ammonia nitrogen as is indicated by the persistence of the high ammonia nitrogen output in the after-period accompanied by an output of chlorine decidedly higher than what is to be expected from the diet, judged by a comparison with the fore-period. Moreover the average total nitrogen excretion of the after-period fails to return to the normal level and in correspondence with this the average minus nitrogen bal-

ance of the after-period was also greater than the normal. Glancing at the creatinine and creatine figures one sees that it was only at the end of the ammonium salt administration that creatine appeared in the urine. The extra ammonia nitrogen eliminated if assumed to be in combination with chlorine would account for the greater portion of the chlorine excreted. It is a little surprising that in these animals with a very small chlorine intake so little of this element should be permanently retained.

These are the facts presented by the data. What interpretation may be placed upon them? It is possible that there may be more than a single explanation but the most obvious one appears to us to be as follows: Ammonium chloride, ingested by the dog, is temporarily retained whatever the state of nutrition, *i.e.*, inanition, maintenance upon an adequate mixed diet or upon a non-nitrogenous ration. If the salt is regarded as possessing a certain degree of toxicity its retention within the body for a short period is not unparalleled since many other compounds of a poisonous nature are likewise temporarily stored. That a toxic action is exerted by ammonium chloride may be concluded from the noticeably increased elimination of total nitrogen in the after-period of both dogs. The appearance of creatine in the urine only after the ammonium salt administration would tend perhaps to point in the same direction according to our present obscure understanding of the function of creatine. To this line of reasoning the objection may be raised that the length of the experiment would be sufficient to account for the increased total nitrogen output after the salt ingestion, *i.e.*, that animals maintained without food nitrogen for such a long period might well show an augmented urinary nitrogen output. Against such an argument may be placed the data in Tables 3 and 4 where the periods are fairly comparable with those in Tables 1 and 2 and yet in which no increased nitrogen excretion is to be observed. Finally, it may be urged that insufficient fuel value in the food was given since less than the calories recommended by Grafe and Schl pfer were supplied. In reply to this objection one needs only to refer to the work of Taylor and Ringer who have demonstrated that carbohydrate intake has little or nothing to do with the retention of the nitrogen of ammonium salts. This fact is also confirmed by the data presented in Tables 3 and 4.

TABLE 1.

## Dog 1.

The daily diet consisted of 60 grams cornstarch, 60 grams sucrose, 60 grams lard, 10 grams bone ash and 600 cc. water. The total fuel value was equivalent to 80 calories per kilo at beginning of experiment when the dog weighed 13 kilos.

DATE 1912	BODY WEIGHT kilos	NITROGEN INTAKE				URINE					Chlorine out- put calcu- lated as Cl	Chlorine intake calculated as Cl	CHLORINE BALANCE PER DAY		NITROGEN IN FEACES		NITROGEN BALANCE PER DAY		REMARKS
		gram	cc.	Specific gravity	Total N grams	Ammonia N mgms.	Creatinine mgms.	Creatinine mgms.	grams	grams	grams	grams	grams	grams	gram	gram	grams	grams	
July																			
10	13.2	0	475	1.006	1.82	0.21	469	0	0.01	0					0.36	-2.18			Fore-period. Standard diet only.
11	13.2	0	510	1.007	1.68	0.21	434	0	0.02	0					0.36	-2.04			
12	13.0	0	560	1.005	1.50	0.19	440	0	0.01	0					0.36	-1.86			
13	13.0	0	550	1.008	1.42	0.20	432	0	0.01	0					0.36	-1.76			
Average	13.1	0	524	1.008	1.60	0.20	444	0	0.01	0					0.36	-1.96			Ammonium chloride pe- riod. In addi- tion to stand- ard diet 3.9 grams ammo- nium chloride = 1.0 gram N were fed per day.
14	13.0	1.0	550	1.010	2.46	0.41	432	0	1.16	2.59					0.45	-1.91			After-period. Standard diet only.
15	12.8	1.0	550	1.011	2.68	0.61	416	0	1.75	2.59					0.45	-2.13			
16	12.7	1.0	655	1.008	2.40	0.78	392	0	2.42	2.59					0.45	-1.85			
17	12.7	1.0	520	1.009	*(1.77)	(0.72)	(300)	0	(1.75)	(2.59)					(0.45)	-1.87			
18	12.6	1.0	610	1.010	2.42	0.91	392	0	2.13	2.59					0.45	-1.94			
Average (4 days)	12.7	1.0	591	1.010	2.49	0.68	408	0	1.86	2.59					0.45	-2.19			
19	12.7	0	520	1.010	1.82	0.70	416	0	2.13	0					0.37	-1.97			
20	12.6	0	390	1.008	1.60	0.41	350	14	0.51	0					0.37	-2.16			
21	12.6	0	445	1.010	1.79	0.33	385	21	0.17	0					0.37	-2.30			
22	12.6	0	535	1.008	2.02	0.35	376	48	0.10	0					0.37	-2.30			

## Dog 8.

The daily diet consisted of 85 grams cornstarch, 85 grams sucrose, 85 grams lard, 10 grams bone ash and 600 cc. water. The total fuel value was equivalent to approximately 80 calories per kilo at the beginning of the experiment when the dog weighed 18.6 kilos.

DATE 1912	BODY WEIGHT	URINE						CHLORINE INTAKE CALCULATED AS Cl	CHLORINE BALANCE PER DAY	NITROGEN IN FECS	NITROGEN BALANCE PER DAY	REMARKS
		Volume	Specific Gravity	Total N	Ammonia N	Creatinine	Creatinine					
	kilos	cc.		grams	gram	mgms.	mgms.	grams	grams	gram	grams	
July												
10	18.6	340	1.010	1.78	0.32	611	0	0.0	0	0.55	-2.33	
11	18.5	400	1.010	1.57	0.31	568	0	0.10	0	0.55	-2.12	
12	18.5	450	1.010	1.69	0.33	560	0	0.10	0	0.55	-2.24	
13	18.4	560	1.010	1.79	0.32	560	0	0.20	0	0.55	-2.34	
Average	18.6	437	1.010	1.71	0.32	576	0	0.16	2.59	0.55	-2.26	Fore-period. Standard diet only.
14	18.2	610	1.010	2.64	0.57	576	0	1.16	2.59	0.63	-2.27	
15	18.1	680	1.010	2.72	0.92	648	0	2.13	2.59	0.63	-2.35	
16	18.2	630	1.010	2.64	1.14	584	0	2.52	2.59	0.63	-2.27	
17	17.8	720	1.010	2.94	1.20	620	0	2.79	2.59	0.63	-2.30	
18	17.5	640	1.010*	(2.81)	(0.93)	(584)	(176)	(2.59)		0.63		
Average	17.9	664	1.010	2.73	0.96	607		2.15	2.59	0.63	-2.29	Ammonium chloride period. In addition to standard diet 3.9 grams ammonium chloride = 1.0 gram N were fed per day.
(4 days)												
19	17.6	470	1.011	2.54	0.54	560	224	0	0	0.71	-3.25	
20	17.5	535	1.010	2.42	0.48	536	192	0.16	0	0.71	-3.13	
21	17.4	580	1.010	2.30	0.49	536	128	0	0	0.71	-3.01	
22	17.4	445	1.012	2.48	0.43	536	184	0	0	0.71	-3.19	
Average	17.4	507	1.011	2.43	0.58	542	182			0.71	-3.14	After-period. Standard diet only.

\* Animal vomited. N in vomit = 0.19 gram; urine contaminated with vomit.

In the absence of any more reasonable explanation it may be concluded that *ammonium chloride ingested by dogs maintained upon a non-nitrogenous diet is in part temporarily retained. This retention is accompanied by evidences of toxicity. Our experiments, therefore, fail to support the contention of Grafe and Schläpfer that the nitrogen of this salt may be utilized as a source of nitrogen supply. On the contrary, they furnish evidence that the ingestion of ammonium chloride may be regarded as a distinct detriment to nutritional rhythm.*

*Experiments with ammonium acetate and citrate.*

To determine whether in our hands other ammonium salts would yield results in harmony with those of Grafe and Schläpfer experiments have been carried out with ammonium salts of organic acids, namely, the acetate and the citrate.

Only a glance at Tables 3 and 4 is necessary in order to glean that these salts show a behavior totally unlike that yielded by ammonium chloride. These protocols give evidence of a considerable daily retention of nitrogen which fails to reappear during the after-period. There is no evidence of a toxic influence and indeed the figures for the after-period indicate a distinct *sparing* influence upon nitrogenous metabolism. Throughout the entire period in both instances creatine was always present in the urine. Its significance under these circumstances is not clear since the degree of its appearance in the two experiments is directly contradictory. Upon comparison of the effects of the two salts it would appear that considerably more nitrogen in the form of the citrate was retained than was true for the acetate. These experiments also emphasize that in any consideration of ammonium salts from the standpoint of intermediary metabolism a distinction must be made between salts of organic acids and those of inorganic acids. To again draw attention to this fact it is necessary only to point out the influence of a single day's ingestion of ammonium chloride (Table 4, October 23) upon the output of ammonia nitrogen. Ingestion of ammonium acetate or citrate has little or no effect upon ammonia nitrogen excretion. When, however, a comparable amount of nitrogen in the form of ammonium chloride is given the ammonia nitrogen elimination is mark-



TABLE 3.  
Dog 3.  
The daily diet was identical with that of Dog 1 in Table 1.

DATE 1912	BODY WEIGHT kilos	NITROGEN INTAKE grams	URINE				NITROGEN IN FEACES gram	NITROGEN BAL- ANCE PER DAY grams	REMARKS
			Volume cc.	Specific Gravity	Total N grams	Ammonia N gram			
October									
22	13.0	0	350	1.012	2.17	0.22	50	-2.51	
23	12.6	0	370	1.015	2.41	0.22	76	-2.75	Fore-period.
24	12.6	0	680	1.008	2.03	0.19	67	-2.37	
Average		0	460	1.012	2.20	0.21	64	-2.54	
25	12.4	1.25	650	1.011	2.70	0.20	47	-1.68	
26	12.4	1.25	560	1.015	2.71	0.21	85	-1.69	10 grams of ammonium citrate = 1.25 grams N were fed per day.
27	12.4	1.25	600	1.010	2.63	0.19	82	-1.61	
Average		1.25	603	1.012	2.68	0.20	71	-1.66	
28	12.4	0	570	1.006	1.92	0.14	156	-2.20	
29	12.2	0	540	1.012	1.57	0.17	116	-1.87	After-period.
30	12.2	0	400	1.013	1.66	0.15	145	-1.96	
Average		0	503	1.010	1.72	0.16	139	-2.02	

TABLE 4

Dog 1.

The diet was identical with that detailed in Table 1.

DATE 1912	BODY WEIGHT		URINE				CREATININE		NITROGEN IN FEACES		NITROGEN BALANCE		REMARKS
	kilos	grams	NITROGEN INTAKE	Volume	Specific Gravity	Total N	Ammonia N	Creatinine	mgms.	mgms.	grams	grams	
October				cc.		grams	gram	mgms.					
11	13.6	0	580	1.010	1.92	0.19	378	64	0.51		-2.43		Fore-period.
12	13.6	0	515	1.010	2.02	0.18	372	114	0.51		-2.53		
13	13.2	0	545	1.010	1.56	0.14	384	113	0.51		-2.07		
Average	13.5	0	547	1.010	1.83	0.17	378	97	0.51		-2.34		
14	13.0	1.0	660	1.008	2.64	0.22	433	174	0.32		-1.96		5.64 grams ammonium acetate = 1.0 gram N were fed per day.
15	13.0	1.0	620	1.009	2.48	0.17	405	53	0.32		-1.80		
16	12.8	1.0	610	1.011	2.74	0.15	405	135	0.32		-2.06		
17	12.6	1.0	Urine contaminated with vomitus										
18	12.6	1.0	Urine contaminated with vomitus										
Average	12.8	1.0	630	1.009	2.62	0.18	414	121	0.32		-1.94		
(3 days)													
19	12.6	0	610	1.009	1.78	0.21	379	39	0.36		-2.14		After-period.
20	12.6	0	520	1.014	1.61	0.18	362	42	0.36		-1.97		
21	12.4	0	580	1.009	1.75	0.17	376	52	0.36		-2.11		
Average	12.5	0	570	1.011	1.74	0.19	372	44	0.36		-2.07		

TABLE 4.—(Continued).

DATE 1912	BODY WEIGHT kilos	NITROGEN INTAKE grams	URINE					Creatinine mgms.	Chloride output as NaCl. grams	NITROGEN IN FECS gram	NITROGEN BALANCE grams	REMARKS
			Volume cc.	Specific Gravity	Total N grams	Ammonia N gram	Creatinine mgms.					
October												
22	12.4	0		urine lost	0							
23	12.4	1.0	470	1.013	2.65	0.50			2.28			3.9 grams ammonium chloride = 1.0 gram N = 2.59 grams Cl were fed on this day.
24	12.2	0	280	1.013	1.68	0.29			0.50			
25	12.0	0	580	1.009	1.63	0.25			0.24			
26	12.0	0	490	1.011	1.50	0.19			0.09			
November												
13	13.6	0	320	1.016	1.95	0.21			trace			4.3 grams NaCl = 2.60 grams Cl were fed on this day in 3 por- tions.
14	13.6	0	430	1.011	1.51	0.21			trace			
15	13.4	0	310	1.020	1.57	0.21			2.50			
16	13.2	0	470	1.014	1.53	0.26			0.60			
17	13.2	0	440	1.015	1.62	0.20			0.66			
18	13.0	0	280	1.020	1.78	0.22			0.20			

edly changed, showing a behavior quite similar to that in a dog upon an adequate mixed diet. The excretion of chlorine is also shown. For the sake of comparison an amount of sodium chloride containing approximately the same quantity of chlorine was administered also for a single day. Under these circumstances sodium chloride produced no influence upon nitrogen elimination and the excretion of chlorine was entirely comparable with that after the ingestion of ammonium chloride for one day. All of the ammonium salts tested showed a distinct tendency toward a diuretic action since in every instance the average urine volume of the experimental period was appreciably higher than in either of the other two periods.

#### CONCLUSIONS.

Contrary to the conclusions of Grafe and Schl pfer it is demonstrated that ammonium chloride shows an entirely different behavior from organic ammonium salts ingested by dogs maintained upon a high calorie non-nitrogenous diet. Ammonium chloride fed under the experimental conditions fails to diminish the loss of nitrogen from the body as determined in a fore-period without ammonium chloride addition; in other words, nitrogen was not retained in the sense of Grafe and Schl pfer.

Under these circumstances urinary ammonia nitrogen is markedly increased beyond the average normal output determined for the fore-period. This fact is regarded as indicative of a temporary retention of the salt which may be looked upon as exerting a marked toxic action. Such an influence is evident from the noticeably increased negative nitrogen balance of the after-period when no ammonium salts were added to the diet. The action of ammonium chloride in these experiments coincides with that previously observed in dogs kept upon an adequate mixed diet and also during a period of inanition.

It may therefore be concluded that ammonium chloride under the experimental conditions is incapable of acting as a source of nitrogen supply for the nutritional needs of the body.

A possible explanation for the discrepancy between the results of Grafe and Schl pfer and our own may perhaps be found in the critique of the methods employed by Grafe and Schl pfer.

Ammonium acetate and ammonium citrate fed under conditions identical with those obtaining for ammonium chloride markedly decrease the nitrogen loss of the experimental period, the average of the fore-period being employed as a standard. No evidence was obtained of the final excretion of the retained nitrogen during the after-period, nor was there any indication of an augmented urinary ammonia nitrogen excretion. Our results obtained with ammonium acetate and ammonium citrate corroborate those of previous investigators.

The present investigation again emphasizes that in any consideration of the influence of ammonium salts upon intermediary metabolism a distinction must be recognized between ammonium salts of organic acids and those of inorganic nature. The ability of the organism to dispose of these two types of salts is radically different.



## COMMENTS ON THE COMMUNICATIONS OF FOLIN AND DENIS.<sup>1</sup>

BY EMIL ABDERHALDEN.

(From the Physiological Institute of the University of Halle.)

(Received for publication, June 28, 1913.)

Folin and Denis have put forward a colorimetric method for the estimation of tyrosine. By the use of this method they have recorded yields of tyrosine which are higher than those hitherto obtained from proteins by any investigator. I have been able to show that tryptophane, hydroxytryptophane and hydroxyproline give the same color reaction as tyrosine, thereby proving that Folin and Denis were in error in stating that they had found a quantitative method for the estimation of tyrosine. Their values are much too high.

Folin and Denis have further shown that the non-coagulable nitrogen of the blood is increased when protein or the products of its hydrolysis are administered *per os*. Their most interesting and fundamental studies have been extended to different important problems. Against the conclusions of Folin and Denis we have raised the following objections: Folin and Denis have not proved that the non-coagulable nitrogen was actually in the form of amino-acids, nor have they in any way shown that the amino-acids are not united to form protein in the wall of the intestine.

In order to clearly separate facts from mere inferences Dr. Lampé and I wished first of all to determine whether compounds containing amino groups, but non-coagulable and giving no biuret reaction, were actually present in blood serum. We have furnished the proof of this. But in order to prove satisfactorily that digested protein reaches the blood solely in the form of amino-acids it is necessary to identify these amino-acids as such and estimate them individually.

<sup>1</sup> This *Journal*, xiv, pp. 453 and 457, 1913.

It is regrettable that Folin and Denis have attacked our criticism so vehemently and go so far as to assert that the hypothesis of protein regeneration in the intestinal wall has been recently modified simply for the purpose of retaining the hypothesis. This is not in accord with the facts. It has always been maintained that amino-acids must remain over and above those which undergo synthesis. Furthermore we have always emphasized that this working hypothesis should be abandoned as soon as facts are disclosed which are in opposition to it. I have no wish to take any part in polemical discussions of hypotheses; but Folin and Denis will doubtless agree with us that careful criticism of investigations has always been of value.

I have reluctantly referred to this matter since the form and tone of Folin and Denis's objections might wrongly give rise to the impressions that our criticisms were not made in the most amicable and impersonal spirit.



## ON THE CEREBROSIDES OF THE BRAIN TISSUE.

SECOND PAPER.

By P. A. LEVENE.

(*From the Rockefeller Institute for Medical Research, New York.*)

(Received for publication, July 2, 1913.)

In a previous investigation on the "Cerebrosides of the Brain Tissue" Levene and Jacobs<sup>1</sup> have brought to light the following new facts: first, that cerebrosides differ not only in their behavior towards solvents, but also in their optical activity, and second, that fatty acids entering in the molecule of different cerebrosides are not identical. Namely, it was possible to isolate from the product of hydrolysis of mixed cerebrosides the *d*, *l*, and the *dl* forms of cerebronic acid. A small quantity of an acid analyzing for  $C_{24}H_{48}O_2$  was also observed. However, it was not obtained in absolutely pure state, its quantity was small, and it was therefore regarded as an impurity.

The cerebrosides employed for last year's work were prepared by the method of Parcus. In this method of preparation fresh, not desiccated, brain pulp is boiled with an aqueous barium hydrate solution. Hence there was a possibility that various optical isomers of the cerebroside isolated in the course of last year's work were artificial products formed in course of preparation. Therefore, it was urgent to establish whether or not brain tissue extracted directly with indifferent solvents contained the same isomers.

In the course of the present year investigations were undertaken on the phosphatides of the brain, and in the process of preparation of this substance, there were obtained considerable quantities of cerebrosides. It was concluded to utilize this material for the investigation of the question whether or not the optical isomers of the cerebrosides found in the mixtures obtained by the method of Parcus were of primary or secondary origin.

<sup>1</sup> Levene and Jacobs: this *Journal*, xii, p. 389, 1912.

In the present year's work the material was obtained from desiccated brain tissue. The tissue was dried under diminished pressure, so that complete desiccation took place within very few hours. The dry material was extracted with hot 95 per cent alcohol, each extraction lasting not more than thirty minutes. The deposit formed on cooling of the alcoholic extract served for preparation of cerebrosides.

The material obtained in this manner differed considerably in its general appearance from that obtained by the method of Parcus. The material was separated in a considerable number of fractions, which differed in their solubility.

The fractions also differed in their optical activity. Most of the measurements were made in pyridine solution in a concentration of 6.66 per cent. Under these conditions the fractions were separated into optically active and inactive. The maximum rotation under the given conditions was  $[\alpha]_D = +4.14^\circ$ . The lowest rotation in pyridine solution was  $[\alpha]_D = +3.05^\circ$ .

On hydrolysis of the optically active fractions only cerebronic acid was obtained. It consisted, however, of a mixture of the optical isomers.

Since in last year's work the fatty acids were obtained by saponification of the esters by means of alkali it seemed possible that some racemization took place in course of saponification, and that originally the cerebrosides contain only one active acid. It was, therefore, concluded to test directly the optical activity of the esters obtained on hydrolysis of the cerebrosides. In fact it was this object that stimulated the work in the directions of thorough fractionation of the cerebrosides prior to hydrolysis.

It was found that in this direction the work of last year was substantiated, as there were obtained esters varying in their optical activity and having the composition of cerebronic acid ethyl ester. The highest rotation observed was  $[\alpha]_D = +2.9^\circ$ , the lowest was  $[\alpha]_D = +1.6^\circ$ . On the other hand, from the more soluble and optically inactive fractions of cerebrosides there were obtained also ethyl esters of cerebronic acid, which were optically inactive. Thus all these observations coincide with those of last year and seem to justify the conclusion arrived at in the previous communication. These conclusions seem still further justified by the following observation. When the cerebroside with a rotation

$[\alpha]_D = +4.14^\circ$ , was dissolved according to Thierfelder in chloroform-methyl-alcohol mixture, then the rotation of a 3.6 per cent solution was  $[\alpha]_D = +9.5^\circ$ , and  $[\alpha]_D = +10.7^\circ$  in a 6 per cent solution. Thierfelder's cerebrin had a rotation of  $[\alpha]_D = +6.4^\circ$ — $8.4^\circ$  in a 5 per cent solution, and on hydrolysis yielded only cerebronic acid. Furthermore Thierfelder's kersin gave on hydrolysis 25 per cent of the total fatty acid in form of cerebronic acid and yet it was optically inactive.

Notwithstanding all this, we are inclined to defer decision on the existence of optically isomeric cerebrinides containing cerebronic acid until more data on the subject are obtained. The reasons are the following: The more soluble fractions of the cerebrinides invariably contain an acid of the composition  $C_{24}H_{48}O_2$ . In this respect our observations fully coincide with the discovery of Thierfelder,<sup>2</sup> although the fractionation in our work was carried out by a process totally different from his. Further, two of our samples, which apparently contained only traces of cerebronic acid, and which were optically inactive in a pyridine solution, showed slight dextro-rotation in a Thierfelder's chloroform-methyl-alcohol solution at a temperature just below the boiling point of the solution. Furthermore it was observed that the specific rotation of the cerebrinides is considerably lowered by the lowering of the concentration of the tested solution. Hence, decision on the existence of isomeric cerebrinides of cerebronic acid has to be deferred until it will be possible to separate completely cerebrinides of cerebronic acid from the other cerebrinides. The new facts brought forward by the work of Thierfelder and by us carry no definite evidence either against or in favor of our last year's assumption.

Regarding the nature of the acid  $C_{24}H_{48}O_2$  our observations differ from those of Thierfelder inasmuch as the melting point of the acid obtained by us, the melting point of its ethyl ester and of its lead salt seemed to coincide with those of lignoceric acid.

In this communication only the experiments dealing with the obtaining of the acid  $C_{24}H_{48}O_2$  will be described in detail. The methods of fractionation and the properties of the cerebrinides will be communicated when individual cerebrinides are obtained in their final purity.

<sup>2</sup> Thierfelder: *Zeitschr. f. physiol. Chem.*, lxxxv, p. 35, 1913.

## EXPERIMENTAL PART.

The cerebrine fraction was optically inactive and had the following composition:

0.1139 gram of substance gave on combustion 0.2955 gram of  $\text{CO}_2$ , and 0.1187 gram of  $\text{H}_2\text{O}$ . C = 70.75; H = 11.66 per cent.

0.2000 gram of substance employed for Kjeldahl nitrogen estimation required for neutralization 2.45 cc. of  $\frac{N}{10}$  acid. N = 1.72.

The substance contained neither phosphorus nor sulphur.

*Ethyl ester of the fatty acid.*

EXPERIMENT I. Thirty-six grams of the substance were taken up in 400 cc. of 98 per cent ethyl alcohol to which 20 cc. of sulphuric acid had been added. The mixture was boiled for seven hours with return condenser in a water bath. The reaction product was allowed to remain over night at room temperature of  $25^\circ\text{C}$ . In the morning, the solution was found filled with a mass of glittering scales. These were filtered off on suction. The residue was again taken up in 150 cc. of ethyl alcohol to which 5 cc. of sulphuric acid had been added and the heating repeated as previously for three hours. The reaction product was again allowed to cool at room temperature of  $25^\circ\text{C}$ . over night, and on the following day the mass of glittering scales was again filtered and dried in a desiccator. The weight of this substance was 2.5 grams.

The two mother liquors were combined and placed in a refrigerator at  $-1^\circ\text{C}$ . over night. A second deposit separated in appearance similar to the first. This was again filtered on suction and dried in the desiccator. The weight of the second product was 2.8 grams. The mother liquor from the second deposit was cooled in an ice and alcohol freezing mixture. A gelatinous mass formed which was again filtered on suction and recrystallized out of about 10 cc. of acetone. The sediment formed in acetone was filtered, dried in a desiccator, and amounted to 0.1200 gram.

The first ester was recrystallized out of acetone and dried over night in a vacuum desiccator. It had a melting point of  $56-57^\circ\text{C}$ . and the following composition:

0.1116 gram of substance gave on combustion 0.3216 gram of  $\text{CO}_2$  and 0.1294 gram of  $\text{H}_2\text{O}$ .

EXPERIMENT II. Twenty-five grams of cerebrine, prepared in the same manner as the substance employed in Experiment I, were taken up in 200 cc. of ethyl alcohol containing 10 cc. of sulphuric acid. This substance was treated in the same manner as in the first experiment. The total yield of the ester was 3 grams. It was not possible to obtain any other esters.

0.1168 gram of substance gave on combustion 0.3356 gram of  $\text{CO}_2$  and 0.1398 gram of  $\text{H}_2\text{O}$ .

		Calculated for $\text{C}_{26}\text{H}_{50}\text{O}_5\text{C}_2\text{H}_5$ :	Found:
Experiment I	C.....	78.8	78.59
	H.....	13.1	12.89
Experiment II	C.....	78.8	78.49
	H.....	13.1	13.42

A 10 per cent pyridine solution of the ester was found to be optically inactive.

#### *Free acid.*

In order to obtain the free acid the ester was saponified in the usual way by dissolving the ester in ethyl alcohol and adding an excess of aqueous sodium hydrate solution. The solution was boiled with return condenser on the water bath for four hours. The reaction product was poured into acetone, washed repeatedly with acetone, then suspended in ether, shaken vigorously for some time, filtered, and the operation repeated several times. The sodium salt obtained in this manner was decomposed with aqueous hydrochloric acid, and placed on the hot water bath until the free acid melted into an oil. The oil was allowed to solidify, the aqueous hydrochloric acid decanted and the free acid freed from hydrochloric acid by repeatedly suspending it in water, melting it on the water bath and allowing the oil to solidify. When the wash water was free from hydrochloric acid the free lignoceric acid was dissolved in a minimum quantity of absolute ethyl alcohol and the equivalent of lead acetate dissolved in methyl alcohol was added. A few drops of aqueous ammonia were then added to complete the precipitation of the lead salt. The mixture was allowed to cool, filtered on suction and washed repeatedly with absolute alcohol. The dry product was dissolved in hot toluene and hydrogen sulphide gas was passed through the solution. The filtrate from the lead sulphide was allowed to

remain in the refrigerator over night. The acid crystallized out. It was then filtered on suction and the last traces of toluene removed by melting the substance on an electric stove. The melting point of this acid was 81°C. Mixed with a sample of an acid obtained on oxidation of cerebronic acid the mixture gave the same melting point.

*Molecular weight of the acid.*

EXPERIMENT I. 0.9566 gram of the acid was dissolved in a mixture of benzin and absolute methyl alcohol and titrated by means of  $\frac{N}{2}$  alkali. It required for neutralization 5.2 cc. of the acid.

EXPERIMENT II. 1.0626 grams of the substance required for neutralization 5.8 cc.  $\frac{N}{2}$  alkali.

	Calculated for $C_{26}H_{48}O_8$ :	Found:
M. W. (Experiment I).....	368	368
M. W. (Experiment II).....	368	366

The ethyl ester of lignoceric acid obtained from other fractions of the cerebrosides, which gave on hydrolysis not only lignoceric but also cerebronic acid, showed some irregularity. Thus the ester, when obtained in exactly the same manner as described in the first two experiments, even though it had been re-esterified twice out of acetone, possessed a melting point of between 60 and 62°C. However, when this ester was saponified and transformed into the free acid which had a melting point of 81°C. and a molecular weight of 36, and was again transformed into the ethyl ester, then the melting point came down to 56–57°C.

*Lead salt of the acid.*

The lead salt was prepared of the pure acid and recrystallized out of a solution consisting of equal parts by weight of benzene and toluene. The melting point of the substance was at 117.5°C.

*Comparison of the constants of lignoceric acid and of the acid obtained from the cerebroside.*

	Lignoceric acid:	Acid from cerebroside:
Free acid, M.P.....	80–81°C.	81°C.
Lead salt, M.P.....	117°C.	117.5°C.
Ethyl ester, M.P.....	55°C.	56–57°C.

**THE INFLUENCE OF PANCREATIC AND DUODENAL  
EXTRACTS ON THE GLYCOSURIA AND THE  
RESPIRATORY METABOLISM OF  
DEPANCREATIZED DOGS.**

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(Received for publication, July 5, 1913.)

In their first contribution on the effects of the complete removal of the pancreas v. Mering and Minkowski<sup>1</sup> reached the conclusion that the diabetes which results can only be accounted for by the cessation of some function of this organ which is necessary for the utilization of sugar. "Utilization of sugar" (*Verbrauch des Zuckers*) in the adult organism means, sooner or later, complete oxidation of sugar. The ultimate proof that it is the ability of the organism to oxidize sugar and not the mere mobilization or availability of sugar which is lacking in the depancreatized animal is found in the two facts of low respiratory quotient and the complete elimination of ingested sugar which are now attested by a number of observers.<sup>2</sup>

Whether the capacity to oxidize sugar is *totally* lost as the result of this operation in dogs need not here detain us. The claim which has been based on the work of Porges and Solomon will be dealt with in a subsequent paper. The important facts are that sugar cannot, under the circumstances, be oxidized in sufficient quantity to be of any service, neither can it be stored (Minkowski); consequently it accumulates in the blood until the kidney barrier is overcome when it appears in the urine.

<sup>1</sup> *Arch. f. exp. Path. u. Pharm.*, xxvi, p. 384, 1889.

<sup>2</sup> Minkowski: *ibid.*, xxxi, p. 85, 1893; Mohr: *Zeitschr. f. exp. Path. u. Ther.*, iv, p. 910, 1907; Falta, Grote and Staehelin: *Hofmeister's Beiträge*, x, p. 199, 1907; Verzár: *Biochem. Zeitschr.*, xlv, p. 201, 1912; Hesse and Mohr: *Zeitschr. f. exp. Path. u. Ther.*, vi, p. 300, 1909.

### 366 Effects of Pancreatic Extracts on Glycosuria

The observation by Knowlton and Starling,<sup>3</sup> confirmed already by McLean and Smedley,<sup>4</sup> that the addition of a pancreatic extract to the blood of a depancreatized dog caused more sugar to disappear from the blood when it was perfused through the beating heart of the depancreatized dog has raised the hope that, at last, the essential pancreatic "hormone" has been discovered. This work cannot, however, be accepted as proving the restoration of pancreatic function until it is shown that the disappearance of sugar from the perfusion fluid denotes actual oxidation of sugar and not polymerization. The disappearance of sugar from a digest containing pancreatic and muscle extracts which Cohnheim<sup>5</sup> reported was shown by Levene and Meyer<sup>6</sup> to be due in reality to a condensation into a polysaccharide. Moreover, according to Elias,<sup>7</sup> a solution of dextrose perfused through the liver of a turtle lost sugar when the fluid was made slightly alkaline and the liver stored glycogen. Knowlton and Starling thus far have excluded neither of these possibilities, the formation of a polysaccharide or the storage of glycogen in the heart muscle.

Loewi<sup>8</sup> has indicated an altogether different type of explanation of Knowlton and Starling's results in his observation that a Locke's solution containing 0.02 per cent of KCl instead of 0.04 per cent caused, when perfused through a diabetic rabbit's heart, the disappearance of as much or more sugar from the fluid than when perfused through the normal heart. Loewi ascribes the effect to the K ion, and alludes to the possibility that sodium ions might drive out potassium.

The final test of such an hormone, however, will be the restoration of pancreatic function to the entire organism. The best criterion of such restoration is the respiratory quotient. Sugar might disappear from the urine and the excess sugar even from the blood after administration of such an extract and still might not be oxidized.

If its oxidation could be restored to normal, the hyperglycaemia should disappear following the disappearance of glycosuria.

<sup>3</sup> *Journ. of Physiol.*, xlv, p. 146, 1912.

<sup>4</sup> *Ibid.*, p. 470, 1913.

<sup>5</sup> *Zeitschr. f. physiol. Chem.*, xlii, p. 401, 1904; xlvii, p. 253.

<sup>6</sup> *This Journal*, ix, p. 97, 1911.

<sup>7</sup> *Biochem. Zeitschr.*, xlviii, p. 120, 1912.

<sup>8</sup> *Münch. med. Wochenschr.*, xiii, p. 690, 1913.



Before Starling and Knowlton's work appeared, the writers had planned some experiments on depancreatized animals with pancreatic and duodenal extracts, the point of departure being an observation by one of them (M.) a few years ago that a mixed glycerin extract of dog's pancreas and duodenal mucosa had, apparently, caused the sugar in the urine of a diabetic man, while he was maintained rigidly on the same diet of milk and eggs, to fall considerably. The observations could not at that time be continued and we had decided upon a series of experiments to test the possibility of a coöperative control of carbohydrate combustion by pancreas and duodenal mucosa.

After a few preliminary experiments with a depancreatized dog in a respiration apparatus constructed on the closed circuit plan of F. G. Benedict,<sup>9</sup> it became apparent that we must study also the effects of the extracts on the glycosuria in order to know at what time we should expect an effect, if any, on the respiratory metabolism.

#### EFFECTS OF EXTRACTS OF PANCREAS AND DUODENUM ON THE D:N RATIO IN DEPANCREATIZED DOGS.

##### *A. Effect of pancreas extracts.*

At first we collected the urine in twenty-four-hour periods. Several such experiments were performed but we shall report only one for the reason that the results were largely negative.

**EXPERIMENT I. Dog I.** A large female pointer weighing 25 kgm. was completely depancreatized by Hédon's<sup>10</sup> technique on March 8, 1912. On March 9-10 the dog had a D : N ratio of 3.26 with a body temperature of 38.4°. On March 11 an extract was made from 670 grams of cow's pancreas brought fresh from the abattoir, the fluid (Ringer's solution) being kept acid with HCl until after boiling. After filtration and neutralization with Na<sub>2</sub>CO<sub>3</sub>, one-half of the total filtrate (500 cc.) was injected subcutaneously in the evening and the other half the following morning.

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<sup>9</sup> *Amer. Journ. of Physiol.*, xxiv, p. 345, 1909.

<sup>10</sup> *Arch. int. de physiol.*, x, p. 350, 1911.

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TABLE I.

DATE March 1913	TIME	TOTAL N	TOTAL D	D : N	TEMP.	REMARKS
	p.m.	grams	grams		°C.	
9	1.00	18.98	62.00	3.26	38.4	Starvation.
10	2.20					
11	2.15	17.41	58.06	3.33	38.2	Injected 600 cc. of pancreatic extract subcutaneously in divided doses, made from 670 grams cow's pancreas.
12	2.20	15.06	57.80	3.83	39.8	One-half injected in evening of March 11. Remainder in morning of March 12.
13	2.20	14.78	52.82	3.57	38.4	Part of urine discarded on account of contamination.
14	2.20	7.33	21.80	2.97	38.4	
15	2.45	5.82	16.20	2.80	38.2	

The only effect which is noticeable in the first twenty-four hours is an increase in the D : N ratio, and it is evident that this is due to a greater fall in the output of nitrogen than in the output of sugar. There was, at the time of catheterization on March 12, a body temperature of 39.8°. The ratio continued high the next day but fell on March 14 and 15. A large abscess had by this time formed in one flank of the dog at the site of injection. This circumstance, although not accompanied by a rise in temperature, may have its bearing on the lower ratio since, as Minkowski<sup>11</sup> observed and as we ourselves have seen in a case of general peritonitis in one of our dogs, a serious infection with formation of pus prevents the appearance of sugar in the urine.<sup>12</sup> It is more likely that the ratios of 2.97 and 2.80 on the last days, however, simply represent an unusually late appearance of the typical ratio.

EXPERIMENT II. *Dog II.* Weight 12.3 kgm.; operated March 24, 1913; without food forty-eight hours before operation. Received no food through-

<sup>11</sup> *Arch. f. exp. Path. u. Pharm.*, xxxi, p. 85, 1893.

<sup>12</sup> Ehrlich long ago discovered that the leucocytes in diabetic blood contain glycogen and Levene and Meyer (this *Journal*, xv, p. 361, 1912), have recently demonstrated the ability of leucocytes to break up glucose *in vitro*.

out the experiment. Qualitative test for sugar immediately after operation. D : N ratio on subsequent day 2.85, temperature 39.2°.

On March 27 the ratio was 3.41 and on March 29, 3.19 in a two-hour period (Table II). Knowlton-Starling extract made from a dog's fresh pancreas was injected intravenously at 4.00-4.20 p.m. This dog, whose pancreas was used, had been fed 100 grams of dextrose one hour before removal of pancreas. The temperature was normal throughout the experiment.

TABLE II.

DATE March 1913	TIME	TOTAL N	TOTAL D	D PER HR.	N PER HR.	D FOR 24 HRS.	N FOR 24 HRS.	D : N PER HR.	D : N PER 24 HRS.	TEMP.								
			grams	grams	grams	grams	grams			°C.								
29	2.30 p.m.	0.84	2.685	1.342	0.42	32.308	10.08	3.19	3.20	38.4								
	4.30 p.m.																	
29	4.00 p.m.	Entire extract of dog's pancreas injected by femoral vein.																
	4.20 p.m.																	
29	4.30 p.m.	1.922	2.937	1.468	0.961			1.52										
	6.30 p.m.																	
29	9.30 p.m.	1.400	4.20	1.400	0.466			3.00		38.2								
30	12.30 a.m.	1.310	3.615	1.205	0.436			2.76		38.2								
30	10.00 a.m.	4.142	12.312	0.880	0.295	25.749	9.614		2.67									
	2.30 p.m.																	
31	2.30	10.44	30.00	1.250	0.420	30.00	10.44		2.97	38.4								

The marked change in the D : N ratio in the period immediately following the injection is plainly due to a rise in the excretion of nitrogen. There is at the same time a smaller increase in the output of sugar which may be due to a washing-out process. In the third period, however, there is, without doubt, a decrease in the sugar which becomes more accentuated in the hourly excretion for the remainder of the twenty-four-hour period. The following twenty-four hours, with the excretion of nitrogen exactly the same as in the fore-period, the sugar returned almost to that level, making a normal Minkowski ratio. No influence of the extract, therefore, is clearly discernible in this experiment.

**EXPERIMENT III.** *Dog III.* Weight 7.8 kgm. Operated April 7, 1913. Without food for forty-eight hours before operation. D : N ratio on the second day following was 2.81. On April 8 a Knowlton-Starling extract was made from six cows' pancreases in 1000 cc. of Ringer's solution. 200 cc. of this extract were injected intravenously after making slightly alkaline with sodium carbonate.

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TABLE III.

DATE April 1913	TIME	TOTAL N	TOTAL D	N PER HR.	D PER HR.	N FOR 24 HRS.	D FOR 24 HRS.	D : N PER HR.	D : N FOR 24 HRS.	TEMP.								
	p.m.	grams	grams	grams	grams	grams	grams			°C.								
8	3.40	1.12	3.40	0.33	0.927			2.81		38.5								
	7.20																	
8	6.10	200 cc. extract of cow's pancreas injected by femoral vein.																
	7.20																	
8	7.20	1.19	3.125	0.274	0.721			2.62		38.2								
	11.40																	
8	11.40	5.08	12.40	0.317	0.775	7.39	18.925	2.44	2.56	38.2								
9	3.30																	
10	3.30	6.66	18.908	0.277	0.787	6.66	18.908	2.84	2.83	38.5								

In a short period immediately preceding the injection in this instance, the hourly excretion of nitrogen was 0.33 gram and of sugar 0.927 gram. The D : N ratio was 2.81. The short period immediately following shows a change in the excretion of nitrogen to 0.274 and in the sugar to 0.721; D : N ratio, 2.62. For the remainder of the twenty-four-hour period, i.e., up to 3.30 the next day, the hourly excretion of dextrose rose slightly and that of nitrogen a little more, making a D : N ratio of 2.44. The following twenty-four hours the sugar remained constant but the nitrogen fell off, restoring the Minkowski ratio of 2.84.

The hourly excretion of sugar was lowest immediately following the injection and rose gradually until, on April 10-11, it was 0.810 gram (Table IV). The ratio on this day is low on account of a higher excretion of nitrogen for some unknown cause. The urine was clear of albumin.

*In only one of the three experiments (III) is there any noteworthy effect of the extract of pancreas made by the Knowlton-Starling method on the elimination of sugar and in this case the typical D : N ratio was restored in the second twenty-four hours.*

### *B. Effects of a mixed extract of pancreas and duodenal mucosa.*

It is well known, from the experiments of Bayliss and Starling, that a substance (secretin) can be extracted from the duodenal mucosa in acid media which has a powerfully stimulating effect

on the external secretions of the pancreas. It seemed possible that a similar substance (or secretin itself) might be necessary to activate the pancreatic component which is responsible for the oxidation of sugar in the normal animal and that the two extracts acting simultaneously might restore this capacity to a depancreatized animal.

EXPERIMENT IV. The same dog was used as in Experiment III. The mixed extract was made in the same manner as before from the organs of a dog which one and one-half hours before anaesthetization had been fed 100 grams of dextrose. The entire extract contained in 150 cc. Ringer's solution was given intravenously on April 11, 5.00-6.00 p.m.

TABLE IV.

DATE April 1913	TIME	TOTAL N	TOTAL D	N PER HR.	D PER HR.	N FOR 24 HRS.	D FOR 24 HRS.	D : N PER HR.	D : N FOR 24 HRS.	TEMP.								
	p.m.	grams	grams	grams	grams	grams	grams			°C.								
10	3.30	7.89	19.460	0.328	0.810	7.89	19.460	2.46	2.46	38.5								
11	3.30																	
11	5.00	150 cc. extract of dog's pancreas and duodenum by femoral vein.																
	6.00																	
11	3.30	6.72	1.413	0.268	0.565			2.10		38.5								
	6.30																	
11	6.00	5.68	13.61	0.264	0.633			2.40		36.4								
12	3.30																	

The double extract of pancreas and duodenal mucosa produced a greater effect on the hourly excretion of sugar than the extract of pancreas alone, notwithstanding the fact that a much greater weight of organ was extracted in the former experiment.

It was already apparent from the preceding experiment that the most marked effect of these extracts on the glycosuria is to be expected immediately after the injection. It was suggested also that when the sugar elimination for the twenty-four-hour period is reckoned up it might be found that the extract has caused only a temporary holding back of the sugar. Accordingly, in the next experiment, the urine was collected in short periods definitely delimited, as all our periods have been, by drawing the urine with the catheter and rinsing out the bladder with sterile water; and at the end the twenty-four-hour amounts were summed up.

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EXPERIMENT V. Dog IV. Weight 12.5 kgm. Operated April 17, 1:30 p.m. The D/N ratio on April 18, 1:15 p.m. was 2.66. On April 19 a Karczewski-Heidel extract was made from the pancreas and duodenal mucosae of two dogs anesthetized with chloroform. The entire double extract was contained after filtration in 150 cc. Ringer's solution. No food was given at any time.

TABLE V

DATE	TIME	TOTAL D	TOTAL N	D PER HOUR	N PER HOUR	D/N	D 24 HRS.
April 1913							
18	2:15 p.m.						
19	6:00 p.m.	25.87	10.08	0.932	0.363	2.58	21.66
19	6:00 p.m.	Injected 150 cc. of pancreatic and duodenal extract by femoral vein.					
	7:00 p.m.						
19	6:00 p.m.	1.33	0.572	0.605	0.305	1.98	
	8:12 p.m.						
19	10:12 p.m.	none	0.602	none	0.301		
20	12:23 a.m.	none	0.612	none	0.305		
20	6:00 p.m.	15.810	4.998	0.865	0.282	3.17	17.10
21	12:00 m.	21.730	8.442	1.20	0.460	2.57	
21	6:00 p.m.	6.107					2.87

Here the effect of injection was immediate and resulted in complete disappearance of sugar at the end of two hours from the time injection began or one hour from the time injection was discontinued. Sugar was completely absent for at least four hours and possibly longer. Boiling with HCl did not reveal any reducing substance. At 6:00 p.m. the next day, however, the amount of sugar per hour, reckoning from midnight when the last collection was made, had risen to the level of the fore-period.

The nitrogen after a preliminary fall from 0.363 to 0.305 remained constant for the full twenty-four hours, after which it rose with the sugar and the original ratio of excretion was resumed. The twenty-four-hour periods show a complete compensation.

EXPERIMENT VI. A second experiment identical in all respects with the first was performed on the same dog. The extract again was made from the pancreas and duodenal mucosae of two dogs.

TABLE VI.

DATE April 1913	TIME	TOTAL D	TOTAL N	D PER HOUR	N PER HOUR	D:N	BLOOD SUGAR	VOL. OF URINE
	p.m.	grams	grams	grams	gram		per cent	cc.
21	12.12 } 4.15 }	4.807	2.044	1.202	0.511	2.35	0.128	80
21	5.00 } 5.30 }	Injected 150 cc. of mixed extract intravenously.						
21	4.15 } 6.15 }	1.30	0.672	0.650	0.336	1.93		30
21	6.15 } 7.15 }	0.288	0.329	0.288	0.329	0.87		25
21	8.15	0.406	0.455	0.406	0.455	0.89	0.148	26
21	9.15	0.588	0.483	0.588	0.483	1.21		26
21	10.15	0.507	0.396	0.507	0.396	1.28		22
22	6.06	18.66	6.944	0.933	0.347	2.68		

The preliminary ratio in this experiment was not quite a typical one for a completely depancreatized dog although there can be no doubt that the organ was completely removed. The lower ratio was due to a high nitrogen figure. The absolute fall in the sugar elimination in this instance was even greater than in the previous experiment although the sugar did not completely disappear from the urine. The volume figures for the urine show that there was no diuretic effect. The blood was analyzed for sugar at 4.00 p.m., one hour before injection and at 8.15 p.m., nearly three hours after the injection ceased. The results show a rise from 0.128 to 0.148 per cent. Twenty-four hours after the injection dextrose and nitrogen were again being excreted in the typical ratio for complete pancreatic diabetes.

There are three possible explanations of the decreased elimination of sugar in these experiments; 1, that the permeability of the kidney to sugar has been reduced; 2, that glycogen has been temporarily stored; and 3, that sugar has been oxidized.

1. *Influence of pancreatic extracts on the permeability of the kidney.* De Meyer<sup>13</sup> made the interesting discovery that when a pancreatic extract was added to Locke's solution perfused through an excised kidney, the permeability of the kidney to an excess of sugar in the perfusion fluid was diminished. About the same

<sup>13</sup> *Arch. int. de physiol.*, viii, p. 121, 1909.

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time Vahlen<sup>14</sup> witnessed a decrease in the output of sugar when a preparation of pancreas was administered to phlorhizinized rabbits. G. Bayer<sup>15</sup> made use of these facts in explanation of the effect of pancreatic extract on adrenalin glycosuria and Wohlgemuth<sup>16</sup> invokes the same explanation to account for a hyperglycaemia without glycosuria which results from ligation of the pancreatic ducts. More recently, Scott<sup>17</sup> injected subcutaneously a watery extract of pancreas into normal cats and obtained an increase in the blood sugar. To what constituent of the pancreas these effects are due is not yet known.

The increase in hyperglycaemia which went hand in hand with the reduction of the glycosuria in Experiment VI indicates that we are dealing here with an alteration of renal permeability.<sup>18</sup> In order to account for the *amount of sugar* retained, however, it is necessary to assume either that some glycogen has been stored or that all the tissue fluids of the body contain sugar to the same extent (percentage) as the blood. The latter possibility would be very difficult to prove.

2. *Influence of the reaction of the medium on the storage of glycogen.* That the reaction of the medium may have a marked influence on glycogenesis and glycogenolysis has been established by the work of Macleod and by the recent work of Elias<sup>19</sup> who found (a) a greatly reduced percentage of glycogen in the liver of normal rabbits, (b) considerable sugar in the urine of both rabbits and dogs, and (c) an active mobilization of glycogen from the liver of the turtle, on administration of dilute HCl. Conversely, there was an increase in glycogen in the turtle's liver when glucose contained in a dilute solution of sodium carbonate was perfused through it. In the liver of depancreatized turtles Nishi<sup>20</sup> had previously found a retention of glycogen upon perfusion with Ringer's solution containing glucose and de Meyer<sup>21</sup> had reported the same for the liver of depancreatized dogs, *when pancreatic*

<sup>14</sup> *Zeitschr. f. physiol. Chem.*, lix, p. 194, 1909.

<sup>15</sup> Quoted by Biedl, *Innere Sekretion*, 1913, i, p. 500.

<sup>16</sup> *Berl. klin. Wochenschr.*, 1913, Feb. 24, p. 339.

<sup>17</sup> *Proc. Soc. of Exp. Biol. and Med.*, x, p. 101, 1913.

<sup>18</sup> Or possibly an effect on the colloid combination of sugar in the blood.

Cf. Allen: *Glycosuria and Diabetes*, Boston, 1913, p. 284, *et seq.*

<sup>19</sup> *Biochem. Zeitschr.*, xlviii, p. 120, 1913.

<sup>20</sup> *Arch. f. exp. Path. u. Pharm.*, lxii, p. 170, 1910.

<sup>21</sup> *Arch. int. de physiol.*, ix, p. 1, 1910.



*extract* was added to the perfusion fluid. Macleod and his co-workers, however, have been unable, under carefully controlled conditions, to prevent post-mortem glycogenolysis much less to cause glycogenesis in the perfused livers of warm-blooded animals and Macleod<sup>22</sup> expresses utter disbelief in de Meyer's statement.

The following experiments were made for the purpose of testing the influence of weak alkali and weak acid on the glycosuria of the depancreatized dog.

EXPERIMENT VII. The same dog was used as in Experiment VI. Two days after that experiment Ringer's solution from the same bottle as that supplying the medium for the organ extracts was made alkaline to about the same degree (1 per cent) as the extract had been made, by adding  $\text{Na}_2\text{CO}_3$  in substance and 150 cc. of this fluid were injected intravenously in exactly the same manner as in Experiment VI.

TABLE VII.

DATE	TIME	TOTAL D	TOTAL N	D PER HOUR	N PER HOUR	D:N	VOL. OF URINE					
April 1913												
		grams	gram	gram	gram		cc.					
24	2.19 p.m.	1.647	0.539	0.823	0.269	3.07	22					
	4.19 p.m.											
24	4.26 p.m.	150 cc. of 1 per cent Na <sub>2</sub> CO <sub>3</sub> intravenously.										
	5.15 p.m.											
24	4.19 p.m.	1.647	0.644	0.823	0.322	2.55	60					
	6.19 p.m.											
24	8.19 p.m.	0.466	0.524	0.233	0.262	0.88	24					
25	1.19 a.m.	2.413	1.475	0.480	0.295	1.62	90					

*The dextrose elimination per hour and the D:N ratio reached the same level at the end of four hours as when the extracts were given.*

Certain points of difference, however, may be noticed: 1, the effect is not immediate with the alkaline Ringer's solution alone; 2, the absolute fall in the sugar excretion is not so great; and 3, there is a marked diuretic effect with this solution while there was none with the organ extracts. These differences may prove to be significant, but, for the present, it will be assumed that they are not so important as the likeness of results between Experiments VI and VII.

EXPERIMENT VIII. Dog IV. Allowing two days to intervene, the same dog was next given an injection of 150 cc. of 2 per cent  $\text{Na}_2\text{CO}_3$ . The

<sup>22</sup> *Diabetes*, 1913, p. 124.

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osmotic effect of this solution would be similar to that of the 1 per cent  $\text{Na}_2\text{CO}_3$  in Ringer's solution.

TABLE VIII.

TIME April 26, 1913	D PER HOUR	N PER HOUR.	D : N	VOL. OF URINE
	gram	gram	grams	cc.
10.45-12.45	0.527	0.227	2.31	19
1.30-2.15	150 cc. of 2 per cent $\text{Na}_2\text{CO}_3$ by femoral vein.			
12.45-2.50	0.280	0.301	0.93	120
2.50-4.50	0.191	0.231	0.82	34
4.50-6.50	0.535	0.233	2.30	33

The lower hourly excretion of sugar at the beginning of this experiment is the result of the gradual decline in sugar production which is always seen in progressive starvation following pancreatectomy. In the second two-hour period following infusion the elimination of sugar is even lower than in the previous experiment in which Ringer's solution was used. There is a primary rise in nitrogen elimination the first two hours, after which the same level is maintained as in the fore-period. The diuresis in the first after-period is much greater than in the previous experiment.

EXPERIMENT IX. *Dog V.* Female. Weight 12.1 kgm. Operated May 19, 1913. Dog had fasted for some days before operation. D : N ratio on May 20 at 9.15 a.m., was 2.32. Twenty-four hours later or forty-three hours after operation it was 2.94. At 11.25 on May 21, 200 cc. of a 2 per cent solution of HCl were given by stomach tube. None was regurgitated.

TABLE IX.

TIME May 21, 1913	TOTAL D	TOTAL N	D : N	D PER HOUR	N PER HOUR	VOLUME
	grams	grams		grams	grams	cc.
Control specimen	33.8	11.53	2.94			
9.15 a.m. }						
11.20 a.m. }	4.72	1.40	3.38	2.26	0.672	44
11.25 a.m.	200 cc. 2 per cent HCl given by stomach tube.					
1.25 p.m. }	5.68	1.456	3.90	2.72	0.699	45
1.25 p.m. }						
3.25 p.m. }	5.68	1.41	4.03	2.84	0.705	40
3.25 p.m. }						
5.25 p.m. }	4.38	1.34	3.27	2.19	0.670	45
5.25 p.m. }						
11.15 a.m.* }	33.3	11.03	3.02	1.85	0.613	

\* May 22.

The effect of the HCl was immediate, and continued for four hours after which there was a compensating fall in the sugar elimination. Contrary to the effect of alkali, there was here no diuresis.

Both these experiments, therefore, turn out as would be expected on the hypothesis that mobilization of glycogen is favored by an acid medium and storage of glycogen by an alkaline medium. Final proof that it is an actual transformation of glycogen in the depancreatized dog as Elias has found it for the normal rabbit must wait on glycogen determinations.

In view of the directly opposite character of the results from the two experiments just presented, it is scarcely possible that the mere dilution of the blood with the infused fluid has produced any effect. Nevertheless, in order to leave no doubt on this point, we have controlled as follows:

EXPERIMENT X. *Dog VI.* Operated May 6. D : N ratio May 9, 3.08.

TABLE X.

TIME May 10, 1913	D PER HOUR	N PER HOUR	D : N
<i>p. m.</i>	<i>grams</i>	<i>gram</i>	<i>grams</i>
1.25-2.25	1.25	0.476	2.62
2.25-3.00	150 cc. sterile water introduced by femoral vein.		
2.25-3.25	1.56	0.66	2.36
3.25-4.25	1.25	0.67	1.87
4.25-6.25	1.26	0.59	2.13

After a slight rise in the sugar excretion for the first hour, the hourly elimination is identical with that of the fore-period. The nitrogen elimination is higher after the infusion of water, probably owing to hemolysis; consequently the D : N ratio is lowered.

3. *The oxidation of sugar.* If the pancreas produces a substance the absence of which in the organism deprives the tissues of their power to oxidize dextrose, it should be possible to restore this substance either by extracting the pancreas or by utilizing blood from a normal animal (Hédon, 1892). There are many possible ways of extracting an organ and many of them have already been employed in an effort to isolate or at least to recover the essen-

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tial "hormone" or internal secretion of the pancreas. Lépine,<sup>23</sup> Caparelli,<sup>24</sup> Ausset,<sup>25</sup> Vahlen,<sup>26</sup> Zuelzer,<sup>27</sup> E. L. Scott<sup>28</sup> and others have put forth claims for pancreatic preparations based, for the most part, on a reduction of the glycosuria either in diabetic persons or in depancreatized animals. None of these preparations, however, have been subjected to the essential test of restoring the power to oxidize sugar, and the facts brought to light by de Meyer, Wohlgemuth and Scott<sup>29</sup> regarding the effect of pancreatic substance on the permeability of the kidney or on the hyperglycaemia (which effects are most readily explained by such a renal action) have at once supplied an adequate explanation for any favorable effects on the glycosuria noted and have rendered very discouraging the quest for the true pancreatic "hormone." Knowlton and Starling's report that a substance soluble from fresh pancreas in acidified Ringer's solution had been found which enhanced if it did not fully restore the powers of the diabetic heart to "burn" sugar, came, therefore, as a very welcome announcement. The present writers confess to a feeling of disappointment that the "disappearance" of sugar from the perfusion fluid was not proved to be due to combustion. Instead of attempting to repeat Knowlton and Starling's experiments, however, it was hoped to find the evidence in an even more direct and crucial test, namely, by means of the respiratory quotient determined on the entire animal.

The time when the effect of the extract of pancreas and duodenal mucosa on the glycosuria was maximal having been found, the double extract<sup>30</sup> was injected intravenously into an animal which had been depancreatized two days before and whose D : N ratio and respiratory quotient both demonstrated its inability to burn sugar, and the respiratory quotient was followed beyond the point when the maximal effect on the glycosuria would have appeared.

<sup>23</sup> *Compt. rend. de soc. biol.*, cxiii, p. 1044, 1891.

<sup>24</sup> *Biol. Zentralbl.*, xii, Nos. 18, 19, 1892.

<sup>25</sup> *Semaine medicale*, xv, p. 326, 1825.

<sup>26</sup> *Loc. cit.*

<sup>27</sup> *Berl. klin. Wochenschr.*, xlvi, p. 1209, 1909.

<sup>28</sup> *Amer. Journ. of Physiol.*, xxix, p. 306, 1912.

<sup>29</sup> *Loc. cit.*

<sup>30</sup> The double extract rather than the extract of pancreas was used because of the greater effects on the glycosuria (see pages 371-373).

**EXPERIMENT XI. Dog VI.** Weight 12.1 kgm. Operated May 6, 1913. On May 9 the D : N ratio was 3.08. At 9.00 o'clock the animal was placed in the small calorimeter recently described by Williams<sup>21</sup> and two one-hour periods were obtained with the dog perfectly quiet. The double extract was prepared from the organs of two dogs killed during this preliminary part of the experiment. After the preliminary experiment the dog was removed from the calorimeter and the entire extract of the glands and mucosae contained in 150 cc. of Ringer's solution made slightly alkaline after filtration, was injected by femoral vein. A urine period of an hour and ten minutes, including the injection period of twenty-five minutes, showed the immediate effect of the extract on the glycosuria. In order to make certain of the presence of sugar in the circulation, 20 grams of dextrose were then given by stomach tube, and the dog was returned to the calorimeter.

TABLE XI.

TIME May 9, 1913	CO <sub>2</sub>	O <sub>2</sub>	R. Q.	D : N	D PER HOUR	CAL. OF HEAT PRODUCED	BODY TEMP.	
	grams	grams			grams			
9.45 a.m.	8.62	9.22	0.68			21.73	38.6	
10.45 a.m.								
10.45 a.m.	9.97	10.55	0.69	3.08	1.736	27.12	38.6	
11.45 a.m.								
12.35 p.m.	150 cc. pancreatic and duodenal extract injected intravenously. 20 grams dextrose in 100 cc. H <sub>2</sub> O per os.							
1.00 p.m.				2.70	1.068			
11.55 a.m.								
1.05 p.m.	13.60	14.13	0.70				39.6	
1.50 a.m.								
2.50 p.m.	11.84	11.78	0.73					
2.50 p.m.								
3.50 p.m.	10.16	10.71	0.69		2.57		39.9	
3.50 p.m.								
4.50 p.m.								

It may be objected that a R. Q. taken three, four or even five hours after injection is only sufficient to prove that no sugar has been oxidized within that time and that it still remains possible that the pancreatic hormone would act later. In answer to this we may cite the compensating increase in the excretion of sugar which began between two and three hours after a similar injection in a previous experiment when no sugar was fed (page 373).

The slight rise in the R. Q. from 0.69 to 0.73 the second hour after infusion of the extract might be due to: 1, experimental error,

<sup>21</sup> This *Journal*, xii, p. 317, 1912.

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this variation being about the limit of error with the apparatus used; 2, the  $\text{Na}_2\text{CO}_3$  given with the extract; or 3, the combustion of not more than 1 gram of dextrose in place of an isodynamic quantity of fat. Convincing proof that very little sugar at most could have been oxidized under the influence of the pancreatic substance injected in this experiment is found in the urinary ratios following. Twenty-four hours after the administration of the dextrose the D : N ratio was 2.62 (see page 377). The ratio just before administration was 3.08. Assuming that the ratio would have fallen uniformly if dextrose had not been given the mean output would have been 2.85 grams of dextrose for each gram of nitrogen. The total N for the twenty-four-hour period was 10.92 grams. The excess sugar, therefore, would be found by subtracting  $(10.92 \times 2.85 =)$  31.12 grams from the total urinary sugar which was 50.32 grams. This accounts for 19.2 or all but 0.8 gram of the amount fed and proves again the complete compensation which takes place following the diminished glycosuria (page 372).

#### HEAT PRODUCTION OR TOTAL ENERGY METABOLISM OF THE DEPANCREATIZED DOG.

This dog had been used for calorimetric observations by Professor Graham Lusk one month previous to this experiment. At that time the basal metabolism was determined in three one-hour periods beginning eighteen hours after last food was given. The  $\text{CO}_2$  elimination was 6.83 grams, the  $\text{O}_2$  absorption 6.03 grams and the total heat production 18.37 calories per hour. In the experiment three days after pancreatectomy, when the D : N ratio was 3.08, the heat production had risen to 27.12 calories, an increase of 42 per cent. This agrees exactly with the result of Falta, Grote and Stachelin<sup>32</sup> for a dog which bore a normal body temperature.

#### EFFECT OF NORMAL BLOOD ON THE R. Q. OF A DEPANCREATIZED ANIMAL.

Remembering the favorable effects on the glycosuria in the experiments of Forschbach<sup>33</sup> on two animals joined parabiotically

<sup>32</sup> *Loc. cit.*

<sup>33</sup> *Deutsch. med. Wochenschr.*, xxxiv, p. 910, 1908; *Arch. f. exp. Path. u. Pharm.*, lx, p. 131, 1908.

and in those of Hédon<sup>34</sup> and of Drennan<sup>35</sup> on dogs transfused with normal dog's blood, the writers attempted also to demonstrate the combustion of sugar in a depancreatized dog which had received by direct anastomosis with a normal dog, 200 grams by weight, of the normal dog's blood, a corresponding amount of the diabetic blood having been previously drawn.

EXPERIMENT XII. *Dog VII.* Operated December 10, 1912. D:N ratio on December 12-13, 2.40.

TABLE XII.

TIME December 17, 1912	CO <sub>2</sub>	O <sub>2</sub> *	R. Q.
<i>p. m.</i>	<i>grams</i>	<i>grams</i>	
1.15-2.15	5.66	6.32	0.651
2.18-3.20	5.65	5.82 (Note)	0.706
4.00	Transfused 200 grams normal dog's blood and fed 20 grams of glucose.		
5.00-6.00	6.43	6.35	0.726
6.00-7.06	5.83	6.18	0.685
8.30-9.30	6.25	6.77	0.672
9.30-10.30	5.55	5.62	0.718

\* Variations in the total oxygen absorption are due to variations in the activity of the dog.

Six and one-half hours after transfusion no favorable effect had been observed. The amount of blood transfused was almost enough to cause the death of the donor, a dog of about 8 kgm. The transfusion of normal blood, in order to establish itself as a measure of any practical importance, should be expected to influence favorably the combustion of carbohydrate with much less than the total amount of "hormone" present in the circulation of an animal at any moment, *and to influence it immediately.* Had 1 gram of dextrose been oxidized in the first period after transfusion instead of an isodynamic quantity of fat the CO<sub>2</sub> output would have been increased by 0.41 gram which would have given a R. Q. of 0.78 instead of 0.73.

It is a singular fact that some observers who have reported the favorable effects on the glycosuria obtained by transfusion should not have taken account of the percentage content of sugar

<sup>34</sup> Latest work, *Arch. internat. de physiol.*, xiii, Heft 1, 1913.

<sup>35</sup> *Amer. Journ. of Physiol.*, xxviii, p. 396, 1911.

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in the blood introduced. Sugar appears in the urine only when the hyperglycaemia reaches a certain height. To reduce this hyperglycaemia markedly by substitution of normal blood with its normal content of sugar must obviously reduce the glycosuria for a time, *without, however, necessarily influencing the combustion of sugar.*

### SUMMARY.

1. Intravenous infusion of pancreatic extract made from cow's pancreas by Knowlton and Starling's method raised the D : N ratio on the days immediately following, when the urine was collected in twenty-four-hour periods.

2. When the urine was collected in short periods a similar extract produced a slight fall in the hourly dextrose elimination and in the D : N ratio in the hours immediately following injection.

3. A mixed extract made in the same manner from dog's pancreas and duodenal mucosa produced a greater fall and in one instance complete disappearance of the urinary sugar. The fall, however, was followed in three to ten hours by a compensating increase.

4. A similar quantity of Ringer's solution made alkaline to about the same degree as the medium for the extract, produced an effect on the glycosuria almost identical.

5. A 2 per cent  $\text{Na}_2\text{CO}_3$  solution likewise caused a sharp decline in the excretion of sugar and a 2 per cent HCl solution by stomach tube, produced a sharp increase. The increase in the one case continued for about the same length of time as the decrease in the other and both are probably to be explained by the effects of the medium on glycogenesis and glycogenolysis.<sup>38</sup>

6. It is possible also that the pancreatic extract affected the renal permeability.

7. Neither extract of pancreas alone nor the double extract of pancreas and duodenal mucosa produced, within the time of maximal effect on the glycosuria, any effect on the respiratory quotient which could be interpreted as an index of increased combustion of carbohydrate.

<sup>38</sup> Cf. McLeod: *Diabetes*, 1913, pp. 150 and 183.



8. The total energy production of a depancreatized dog was found to be 42 per cent greater than that of the same dog while normal.

9. Transfusion of 200 grams of normal dog's blood likewise produced no observable effect on the R. Q. of a depancreatized dog.

We conclude that neither the use of the Knowlton-Starling extract nor the transfusion of normal blood is yet a measure of any practical importance in restoring to the depancreatized dog the ability to burn sugar.



# PROTOZOAN PROTOPLASM AS AN INDICATOR OF PATHOLOGICAL CHANGES.

## I. IN NEPHRITIS.

By LORANDE LOSS WOODRUFF AND FRANK P. UNDERHILL.

(From the Sheffield Laboratories of Biology and Physiological Chemistry,  
Yale University, New Haven, Connecticut.)

(Received for publication, June 19, 1913.)

## CORRECTION.

In Vol. XV, No. 2, August, 1913, the last line of Table I, page 307,  
should read as follows:

57*	3.60	2.18	0.29	1.13	2.20	61	8	31
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nated. Accordingly in the present work an attempt is made to  
demonstrate the existence of chemical changes arising from patho-  
logical conditions by determining the effects of normal and patho-  
logical tissue extracts on a unicellular animal, *Paramecium*.

*Paramecium* was adopted as our "biological reagent" because  
the ease with which it lends itself to experimental methods makes  
it one of the most favorable forms for general physiological study;  
and because we had at our disposal a pedigreed race of *Parame-  
cium aurelia* which had been under daily observation for over  
six years or through more than 3700 generations.<sup>1</sup> This pedi-

<sup>1</sup> Woodruff: *Archiv für Protistenkunde*, xxi, p. 263, 1911; *Proc. Soc. Exp.  
Biol. and Med.*, ix, p. 121, 1912; *Biologische Centralblatt*, xxxiii, p. 34, 1913.

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# PROTOZOAN PROTOPLASM AS AN INDICATOR OF PATHOLOGICAL CHANGES.

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Although pathological changes are undoubtedly in most instances the result of chemical disturbances, a simple biological method for demonstrating the existence of such changes has not been devised. The indication of subtle alterations occurring in one organism by, for example, the injection of an extract into another animal is obviously complicated by the highly specialized physiological processes of the vertebrate organism as well as by individual idiosyncrasies of the inoculated individual; and further, marked differences have been found in the toxic action of salts toward various tissues of the same animal, as well as toward the same tissue under different conditions. When, however, the organism is reduced to the lowest possible term, the single cell, these complications are considerably diminished if not entirely eliminated. Accordingly in the present work an attempt is made to demonstrate the existence of chemical changes arising from pathological conditions by determining the effects of normal and pathological tissue extracts on a unicellular animal, *Paramecium*.

*Paramecium* was adopted as our "biological reagent" because of the ease with which it lends itself to experimental methods makes it one of the most favorable forms for general physiological study; because we had at our disposal a pedigreed race of *Paramecium aurelia* which had been under daily observation for over ten years or through more than 3700 generations.<sup>1</sup> This pedi-

Woodruff: *Archiv für Protistenkunde*, xxi, p. 263, 1911; *Proc. Soc. Exp. Biol. and Med.*, ix, p. 121, 1912; *Biologische Centralblatt*, xxxiii, p. 34, 1913.

greed race afforded us an unfailing supply of protozoa whose environment and morphological<sup>2</sup> and physiological condition were accurately known for a long time and which was bred under particularly constant conditions during the period in which our experiments were made. The importance of these factors must be emphasized because numerous studies testify to the fact that the reactions of paramecia to various stimuli are greatly modified by their past and present environment.<sup>3</sup> Greeley, for example, in a study of the effects of various chemicals on the protoplasm of *Paramecium* insisted that "maximum dilutions can only be approximate, as the action of identical solutions is not the same on paramecia from different cultures, because no two are exactly alike in respect to chemical composition and osmotic pressure."<sup>4</sup> Further, since all the organisms we employed were derived originally from one animal, all the experiments were made on "sister" cells, and therefore on the "same protoplasm."

Previous experiments<sup>5</sup> on this race of *Paramecium* have demonstrated that the rate of reproduction is a most accurate indication of the chemical composition and temperature of the environment, as remarkably slight variations in the culture medium produce characteristic responses by the animals. Indeed, the rate of reproduction may be said to be a function of the environment of the cell. Accordingly the present study involves a comparison of the rate of reproduction of subcultures from this pedigreed race of *Paramecium* bred on extracts of normal and of nephritic kidneys.

#### *Methods.*

1. *Conduction of cultures.* To initiate an experiment a single paramecium was isolated from the pedigreed race with a capillary pipette under a Zeiss binocular microscope and placed in a watch glass with a small amount of culture medium (hay infusion) similar to that employed for the main lines of the race. When, in the course of a few days, this animal by division had

<sup>2</sup> Woodruff: *Journ. of Morphology*, xxii, p. 223, 1911.

<sup>3</sup> Woodruff: *Biol. Bull.*, xxii, p. 60, 1911.

<sup>4</sup> Greeley: *Ibid.*, vii, p. 1, 1904.

<sup>5</sup> Woodruff: *Biochem. Bull.*, i, p. 396, 1912; Woodruff and Bunsel: *Amer. Journ. of Physiol.*, xxv, p. 190, 1909; Woodruff and Baitzell: *Ibid.*, xxix, p. 147, 1911.

produced a sufficient number of cells for the experiment each of the organisms was isolated on a clean depression slide and supplied with five drops of the extract to be tested.<sup>6</sup> Depression slides containing the eight lines of animals subjected to the same extract were placed in a moist chamber to prevent evaporation. At the start of the experiment and also at the time of the daily isolations precautions were taken to secure the inoculation with an identical bacterial flora of the extracts to be compared. The glassware employed was boiled in water redistilled from glass. All the organisms whose rate of reproduction was directly compared were bred at the same time and at the same room temperature. Consequently fluctuations in temperature affected all simultaneously and therefore need not be considered.

Each extract was tested on eight lines of sister cells for five days. Every day a single animal was isolated from each of the lines, placed on a clean depression slide and supplied with five drops of the extract from the test tube containing that day's supply. At the time of isolation a record was made of the number of divisions in each of the eight lines of cells during the previous twenty-four hours and these data are the basis of the results submitted in the present paper.

2. *Preparation of extracts.* In the preparation of kidney extracts extreme care was taken to exclude all possible sources of contamination. For the accomplishment of this object all vessels employed were of glass, usually of Jena make, and were used for no other purpose. These receptacles were cleaned with ordinary distilled water which had been redistilled in glass. Similar water was used in the preparation of the extracts. The technique employed in the preparation of the kidney extracts was as follows: The experimental animals were killed by bleeding, the kidneys immediately excised, dissected free from adhering fat, separately ground to a hash in a small meat chopper, and treated with a weight of water equal to five times the weight of the kidney. This mixture was slowly brought to the boiling point in an Erlenmeyer flask, covered with a watch glass to prevent undue evaporation, and maintained at this temperature for a period of five minutes with frequent shaking. The mixtures were then filtered.

<sup>6</sup> Woodruff: *Journ. of Exp. Zoölogy*, x, p. 557, 1911.

In the preliminary experiments the filtration was made upon a folded filter, the solution being repeatedly passed through the paper to obtain a clear solution. In later work this was accomplished by filtering through a thick mat of washed filter pulp. In some instances neither type of filtration afforded more than an approximation to a perfectly clear solution. This was especially true for the extracts made from the nephritic kidneys. The filtered solutions for immediate use were placed in small test tubes, quantities sufficient for a day being employed. The surplus fluid was placed in small Erlenmeyer flasks. In either event the vessels were stoppered with cotton plugs, sterilized in an autoclave and kept in sealed jars in a dark, cool closet.

*The behavior of Paramecium toward extracts of normal kidneys of the same and different animals.*

It is obvious that the method of employing *Paramecium* as an indicator of pathological chemical changes must necessarily depend upon at least two conditions. In the first place it is conceivable that this organism may be so sensitive to slight chemical alterations as to render the method valueless; that, for instance, extracts of normal kidneys may vary sufficiently in composition to elicit a corresponding response on the part of *Paramecium*. On the other hand it is possible that, with the technique employed in the preparation of the extracts, the solutions finally used are not identical chemically. In other words, that two extracts prepared from normal kidneys of the same animal or of different animals will possess noticeably different composition.

To determine the degree of variability in the composition of such extracts as detected by *Paramecium* the following experiments have been carried through.

EXPERIMENT 1. The female rabbits, of approximately 2000 grams' weight each, selected for this experiment had been maintained under identical conditions for a period of at least three months and were in a state of good nutrition. The kidneys of Rabbit I each weighed 6.5 grams; those of Rabbit II, 5.0 grams. Extracts of each kidney made in the manner previously outlined showed a neutral reaction to litmus. These extracts were diluted with water in the ratio of one part of extract to three parts of water and were then employed as culture media for *Paramecium* of the pedigreed race already described, with the results recorded in Table I.



TABLE I.

*The effects on the division rate of Paramecium of the extracts of normal kidneys of the same and of different rabbits.*

		CULTURE A					CULTURE B					TOTAL DIVISIONS FOR BOTH CULTURES
LINE	NO. OF DIVISIONS AFTER DAYS					NO. OF DIVISIONS AFTER DAYS						
	1	2	3	4	5	1	2	3	4	5		
<i>Rabbit I, Kidney A (normal).</i>												
1	1	5	8	11	15	3	5	7	11	15		
2	2	5	8	11	15	3	5	8	11	15		
3	2	5	8	10	14	2	5	7	10	14		
4	3	6	8	11	15	3	5	8	10	14		
Totals.....	8	21	32	43	59	11	20	30	42	58	117	
<i>Rabbit I, Kidney B (normal).</i>												
1	3	6	9	12	16	2	5	8	12	15		
2	3	6	8	12	15	3	6	9	12	16		
3	3	7	9	12	16	3	5	8	11	15		
4	2	5	8	12	15	2	4	7	11	15		
Totals.....	11	24	34	48	62	10	20	32	46	61	123	
<i>Rabbit II, Kidney A (normal).</i>												
1	3	6	9	12	17	3	6	8	11	15		
2	3	5	8	12	15	2	5	8	10	14		
3	3	6	9	12	16	2	5	8	11	14		
4	3	6	8	12	16	2	5	8	11	15		
Totals.....	12	23	34	48	64	9	21	32	43	58	122	
<i>Rabbit II, Kidney B (normal).</i>												
1	3	6	8	12	16	0	4	6	9	13		
2	2	6	8	11	15	3	6	8	11	15		
3	3	6	8	11	16	3	6	8	12	15		
4	3	6	8	12	15	3	6	8	11	15		
Totals.....	11	24	32	46	62	9	22	30	43	58	120	

Inspection of the data will show that *Paramecium* fails to indicate any chemical difference in the extracts of the kidneys of the same animal or of different individuals as judged by the division rate of *Paramecium*, the variations occurring being within the limits of error of the experiment. It may therefore be concluded that these extracts possess a similar chemical composition, and further that the technique employed in their preparation is sufficiently accurate for the purposes of this investigation.

*The influence of extracts of nephritic kidneys upon the division rate of Paramecium.*

For testing the division rate of *Paramecium* toward pathological tissue extracts, use has been made of kidney extracts obtained from animals with tartrate nephritis. It has been shown<sup>7</sup> that subcutaneous injection into rabbits of sodium tartrate causes marked histological changes in the convoluted tubules, accompanied by noticeable variations in the elimination of the urinary constituents. It is possible that these morphological changes are related to chemical alterations which may perhaps contribute directly to the almost invariable fatal outcome observed; although it has by no means been established that in nephritis toxic products are produced. At any rate this experimental condition affords an excellent opportunity to test the sensitiveness of our proposed biological indicator of chemical change.

During tartrate nephritis animals refuse food, hence it became necessary to test the influence of starvation alone as a control upon the reaction under discussion. This has been done in the experiment to be detailed below.

**EXPERIMENT 2.** Three female rabbits, kept under conditions identical with those of Experiment 1, were placed in metabolism cages. Rabbit III weighing 1900 grams, was subjected to simple starvation. Rabbits IV and V, each weighing 2500 grams, received two injections of 0.5 gram sodium tartrate respectively two days apart. No food was given. All three animals received a sufficiency of water. At the end of the third day the animals receiving the tartrate secreted the scanty colorless urine typical of nephritis and all the animals were killed by bleeding. Autopsy revealed the characteristic tartrate kidneys in Rabbits IV and V. The combined

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<sup>7</sup> Underhill: this *Journal*, xii, p. 115, 1912; Underhill, Wells and Goldschmidt: *Journ. of Exp. Med.*, xviii, Sept., 1913.

kidneys weighed as follows: Those of Rabbit III = 11.0 grams; those of Rabbit IV = 22 grams; those of Rabbit V = 25 grams. It will be observed that the nephritic kidneys were twice as heavy as those of the animal that had been subjected to starvation only. The softness of these tissues would lead one to infer that the increased weight was due to accumulated water. In order to make allowance for this possibility the extracts were suitably diluted, the details being given below.

In the preparation of the kidney extracts the organs of each animal were combined and treated in the manner noted under the section on methods. The extract obtained from Rabbit III was clear and definitely yellow in color. Those from Rabbits IV and V could not be filtered clear upon an ordinary paper and were quite turbid. The filtrates from the kidneys of Rabbits IV and V were divided into two portions, one of which was diluted with three times its volume of water, the other with one and one-half times its volume of water. The latter dilution was made on the assumption that the marked difference in weight in the kidneys noted above was due to water. The extracts were labeled as follows:

A = extract obtained from Rabbit III.

B = extract obtained from Rabbit IV.

C = extract obtained from Rabbit V.

TABLE II.

*The effects upon the division rate of Paramecium of nephritic kidney extracts.*

RABBIT	CULTURE	NO. OF DIVISIONS AFTER DAYS				
		1	2	3	4	5
I. Normal	A.....	4	17	32	45	53
	B.....	7	21	33	45	53
	Total.....	11	38	65	90	106
III. Starvation, ext. A	A.....	8	20	33	46	53
	B.....	8	20	33	44	52
	Total.....	16	40	66	90	105
IV. Nephritis, ext. B	A.....	8	15	30	42	49
	B.....	7	12	25	37	44
	Total.....	15	27	55	79	93
V. Nephritis, ext. C	A.....	5	13	25	37	43
	B.....	5	15	27	39	45
	Total.....	10	28	52	76	88

In each instance one part of the original extract was diluted with three times its volume of water.

The portions of the original extracts that were diluted with one and one-half times their volume of water were designated:

B' = extract obtained from Rabbit IV.

C' = extract obtained from Rabbit V.

In Tables II and III will be found the results obtained with these extracts when tested toward *Paramecium*. Table II contains the data furnished by the use of extracts A, B and C, when compared with an extract obtained from a normal kidney (Rabbit I, Experiment 1). The data in Table III were derived by the employment of extracts A (simple starvation), B' and C' and a normal kidney extract (Rabbit I, Experiment 1).

Comparison of the figures in Table II shows that: 1. The extract obtained from the kidney of an animal subjected to inanition for a period of three days does not differ from that of a normal kidney extract when tested toward *Paramecium*. 2. Nephritic

TABLE III.

*The effect upon the division rate of Paramecium of nephritic kidney extracts.*

RABBIT	CULTURE	NO. OF DIVISIONS AFTER DAYS				
		1	2	3	4	5
I. Normal	A.....	2	7	14	19	34
	B.....	1	8	13	15	32
	Total.....	3	15	27	34	66
III. Starvation, ext. A	A.....	3	8	12	18	31
	B.....	3	9	15	19	29
	Total.....	6	17	27	37	60
IV. Nephritis, ext. B'	A.....	0	3	6	13	26
	B.....	0	4	7	9	19
	Total.....	0	7	13	22	45
V. Nephritis, ext. C'	A.....	0	0	3	5	17
	B.....	0	1	4	4	16
	Total.....	0	1	7	9	33

kidney extracts cause a marked depression of the division rate of *Paramecium*.

On the assumption that the extra weight of the kidneys in Rabbits 4 and 5 was due to water, the comparative influence of extracts B' and C' was tested (see Table III). Inspection of the data here presented makes it evident that the depression of division rate noted in Table II cannot be ascribed to mere dilution, for the same type of action is even more markedly demonstrated in spite of the greater concentration of the pathological kidney extracts.

*It may therefore be concluded that under our experimental conditions Paramecium is capable of distinguishing between normal kidney extracts and those obtained from kidneys rendered abnormal by sodium tartrate.*

*The behavior of Paramecium toward solutions of sodium tartrate.*

There is a possibility that the depressing effect of nephritic kidney extracts may be explained by the presence in these extracts of significant quantities of tartrate itself. This suggestion appears very reasonable in view of the fact that tartaric acid is not readily burned in the organism and when subcutaneously administered is not eliminated to any extent. The specific action of tartrate upon the convoluted tubules also lends support to the assumption that a considerable portion of the injected tartrate may accumulate in the kidneys, and hence produce a depressing action upon *Paramecium* when the latter is placed in the kidney extract.

To determine the influence of various strengths of tartrate upon the division rate of *Paramecium*, pure recrystallized sodium tartrate was dissolved in a solution of beef extract.<sup>8</sup> As a control beef extract without tartrate addition was employed.

From Tables IV and V it may be observed that strengths of tartrate below 0.1 per cent exerted little or no influence upon the rate of reproduction of the paramecia whereas 0.1 per cent solutions of tartrate caused a slight stimulation. The data in Table V demonstrate that sodium tartrate in 0.2 per cent solu-

<sup>8</sup> Woodruff and Baitsell: Beef Extract as a Constant Culture Medium for *Paramecium*, *Journ. of Exp. Zoölogy*, xi, p. 135, 1911.

tion also has a stimulating effect, whereas in solutions of the strength of 0.3 per cent a marked depressant influence may be observed, resulting in death within five days. When tartrate solutions stronger than 0.3 per cent were employed the organism was quickly killed.

TABLE IV.

*The effects upon the division rate of Paramecium of various strengths of sodium tartrate dissolved in beef extract.*

SUBSTANCE TESTED	CULTURE	NO. OF DIVISIONS AFTER DAYS				
		1	2	3	4	5
Beef ext. (normal)	A.....	3	12	22	28	34
	B.....	2	7	19	23	26
	Total.....	5	19	41	51	60
Beef ext. + tartrate (0.005 per cent)	A.....	3	11	22	29	36
	B.....	2	10	18	25	32
	Total.....	5	21	40	54	68
Beef ext. + tartrate (0.001 per cent)	A.....	2	9	21	31	35
	B.....	3	6	18	26	32
	Total.....	5	15	39	57	67
Beef ext. + tartrate (0.01 per cent)	A.....	3	8	18	23	30
	B.....	3	10	17	21	27
	Total.....	6	18	35	44	57
Beef ext. + tartrate (0.03 per cent)	A.....	3	6	18	24	30
	B.....	2	6	19	28	33
	Total.....	5	12	37	52	63
Beef ext. + tartrate (0.1 per cent)	A.....	2	8	22	31	41
	B.....	5	8	20	30	39
	Total.....	7	16	42	61	80

The same type of test was applied to *Paramecium* employing normal kidney extract to which definite quantities of sodium tartrate had been added. In Table VI is shown the influence of

normal kidney extracts containing 0.025, 0.05, and 0.1 per cent sodium tartrate, and the conclusion may be drawn that tartrate in the strengths given above causes a slight stimulating action. It has been determined that from 0.2 per cent through 0.5 per cent sodium tartrate in normal kidney extract exerts a depressing influence. Comparison of this conclusion with that derived from comparable strengths of tartrate in beef extract shows that in the beef extract 0.1 and 0.2 per cent sodium tartrate has a stimulating influence. This behavior may be taken as a further proof

TABLE V.

*The effects upon the division rate of Paramecium of various strengths of sodium tartrate dissolved in beef extract.*

SUBSTANCE TESTED	CULTURE	NO. OF DIVISIONS AFTER DAYS				
		1	2	3	4	5
Beef ext. (normal)	A.....	4	7	8	9	12
	B.....	4	6	9	10	11
	—	—	—	—	—	—
	Total.....	8	13	17	19	23
Beef ext. + tartrate (0.2 per cent)	A.....	8	11	15	16	18
	B.....	7	12	16	17	23
	—	—	—	—	—	—
	Total.....	15	23	31	33	41
Beef ext. + tartrate (0.3 per cent)	A.....	5	8	10	10	
	B.....	6	10	10	10	
	—	—	—	—	—	—
	Total.....	11	18	20	20	Dead

of the sensitiveness of paramecia as an agent to detect chemical differences in their environment. When sodium tartrate in concentrations greater than 0.5 per cent was used the organisms were killed within forty-eight hours, and in some cases, for example with concentrations as high as 0.7, 0.8 and 0.9 per cent, in less than twenty-four hours.

From these observations it is clear that if the depressing effect of nephritic kidney extracts is to be ascribed to the presence of sodium tartrate, these solutions must contain the salt to the extent of at least 0.2 per cent. Such a possibility is exceedingly remote.

Nevertheless, in order to determine whether appreciable quantities of tartrate were present, tests for it were made by precipitation with calcium chloride. Preliminary trials with solutions of pure tartaric acid demonstrated that no precipitation occurred with calcium in solutions of less than 0.25 per cent. Extracts of the nephritic kidneys employed above failed to show any reaction with calcium. When these extracts were concentrated to one-

TABLE VI.

*The effects upon the division rate of Paramecium of various strengths of sodium tartrate dissolved in normal kidney extract.*

SUBSTANCE TESTED	CULTURE	NO. OF DIVISIONS AFTER DAYS				
		1	2	3	4	5
Kidney ext. (normal)	A.....	11	19	27	37	41
	B.....	12	18	27	37	41
	—	—	—	—	—	—
	Total.....	23	37	54	74	82
Kidney ext. + tartrate (0.1 per cent)	A.....	13	21	31	43	47
	B.....	11	20	31	42	46
	—	—	—	—	—	—
	Total.....	24	41	62	85	93
Kidney ext. + tartrate (0.05 per cent)	A.....	12	20	31	41	47
	B.....	12	21	31	43	46
	—	—	—	—	—	—
	Total.....	24	41	62	84	93
Kidney ext. + tartrate (0.025 per cent)	A.....	13	20	30	40	43
	B.....	14	21	33	44	46
	—	—	—	—	—	—
	Total.....	27	41	63	84	89

fifth their volumes the reaction was still negative. It is probable, therefore, that if tartrate was present in these extracts it must have been in a concentration less than 0.05 per cent tartaric acid. If this conclusion is correct it must be accepted that the depressing influence of nephritic extracts noted cannot be ascribed to tartrate, since concentrations of this salt in kidney extracts up to 0.1 per cent cause stimulation.



Although it may be granted that the presence of tartrate in the kidney extracts is not responsible for the depressant effect observed upon the rate of division of *Paramecium*, the problem has been attacked from another standpoint. It has been demonstrated that large doses of tartrate do not necessarily produce a more severe type of nephritis than small quantities;<sup>9</sup> nevertheless it is reasonable to assume that, if tartrate does accumulate in the kidney, more would be present in extracts made from kidneys of animals receiving a large injection of tartrate than in kidney extracts prepared from rabbits to whom very small quantities of this salt has been administered. Under these circumstances it is evident that the extracts presumably containing the larger quantities of tartrate should exert a much greater influence upon the rate of division of the paramecia than might be anticipated from extracts with a small tartrate content. To determine the influence of extracts made under these conditions the observations noted below have been carried out.

**EXPERIMENT 3.** Five white female rabbits, each weighing approximately 1500 grams, and which had been kept for a reasonable period under identical conditions, were placed in metabolism cages. The animals were designated Rabbits VI, VII, VIII, IX and X. Rabbit VI was kept for a period of four days without food to serve as a control, no tartrate being administered. Rabbits VII and VIII each received 0.5 gram sodium tartrate after a two days' period of starvation, and under the same conditions 2.0 grams of sodium tartrate were administered to Rabbits IX and X. The preliminary two days' period of inanition was allowed in order to render the animals as sensitive as possible to the influence of tartrate.<sup>10</sup> Sufficient water was given to each animal. In all four cases the typical symptoms—either colorless urine or anuria—prevailed at the end of the second day subsequent to tartrate injection. At this time the animals were killed by bleeding. The combined kidneys of each animal weighed as follows: Rabbit VI (normal) = 11.0 grams; Rabbit VII (very pale and soft) = 10.0 grams; Rabbit VIII (pale and soft) = 11.0 grams; Rabbit IX (pale and soft) = 15.0 grams; Rabbit X (very much congested) = 16.0 grams. Extracts of these kidneys were made in the usual manner. Considerable difficulty was experienced in preparing perfectly clear extracts but this object was finally attained by filtering through a heavy mat of filter pulp. It will be noted that kidneys of animals receiving the large dose of tartrate weighed considerably more than the others.

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<sup>9</sup> Underhill, Wells and Goldschmidt: *loc. cit.*

<sup>10</sup> *Ibid.*

The influence of these solutions upon the division rate of *Paramecium* may be seen in Tables VII and VIII. These figures make it evident that if more tartrate does accumulate in the kidneys of animals receiving the larger dose of tartrate there is no evidence of

TABLE VII.

*The effects upon the division rate of Paramecium of nephritic kidney extracts obtained from rabbits receiving small and large doses of sodium tartrate.*

RABBIT	CULTURE	NO. OF DIVISIONS AFTER DAYS				
		1	2	3	4	5
VI. Normal (1:3)	A.....	8	17	28	37	47
	B.....	11	19	33	43	54
	—	—	—	—	—	—
	Total.....	19	36	61	80	101
VII. Nephritis, small dose tartrate (1:3)	B.....	8	14	23	31	39
	B.....	8	17	29	36	43
	—	—	—	—	—	—
	Total.....	16	31	52	67	82
VIII. Nephritis, small dose tartrate (1:3)	A.....	9	19	31	38	48
	B.....	9	17	30	38	45
	—	—	—	—	—	—
	Total.....	18	36	61	76	93
IX. Nephritis, large dose tartrate (1:3)	A.....	7	15	26	31	43
	B.....	8	15	27	33	43
	—	—	—	—	—	—
	Total.....	15	30	53	64	86
IX. Nephritis, large dose (1:2.25)	A.....	8	17	26	33	41
	B.....	6	14	26	31	40
	—	—	—	—	—	—
	Total.....	14	31	52	64	81
X. Nephritis, large dose (1:3)	A.....	8	15	27	35	46
	B.....	8	16	29	35	46
	—	—	—	—	—	—
	Total.....	16	31	56	70	92
X. Nephritis, large dose (1:2.25)	A.....	8	14	28	32	41
	B.....	7	14	26	31	42
	—	—	—	—	—	—
	Total.....	15	28	54	63	83

it indicated by the behavior of paramecia bred in such kidney extracts. At any rate paramecia do not show any different behavior when placed in kidney extracts of animals receiving large doses of tartrate than may be observed under similar conditions

TABLE VIII.

*The effects upon the division rate of Paramecium of nephritic kidney extracts obtained from rabbits receiving small and large doses of sodium tartrate.*

RABBIT	CULTURE	NO. OF DIVISIONS AFTER DAYS				
		1	2	3	4	5
VI. Normal (1:3)	A.....	10	20	30	41	50
	B.....	9	20	30	42	51
	Total.....	19	40	60	83	101
VII. Nephritis, small dose tartrate (1:3)	A.....	5	12	19	26	34
	B.....	5	13	19	27	33
	Total.....	10	25	38	53	67
VIII. Nephritis, small dose tartrate (1:3)	A.....	11	20	28	39	47
	B.....	8	17	27	37	45
	Total.....	19	37	55	76	92
IX. Nephritis, large dose tartrate (1:3)	A.....	6	14	23	32	42
	B.....	4	12	22	30	39
	Total.....	10	26	45	62	81
IX. Nephritis, large dose tartrate (1:2.25)	A.....	6	13	20	29	39
	B.....	7	14	21	29	37
	Total.....	13	27	41	58	76
X. Nephritis, large dose tartrate (1:3)	A.....	5	14	22	33	42
	B.....	7	16	24	34	44
	Total.....	12	30	46	67	86
X. Nephritis, large dose tartrate (1:2.25)	A.....	7	16	24	34	43
	B.....	4	14	21	30	39
	Total.....	11	30	45	64	82

with kidney extracts of animals into whom only small quantities have been injected. It is therefore very improbable that the quantity of tartrate which perhaps may be present in kidney extracts of tartrate-injected animals plays any significant rôle in the depressant effect observed upon the division rate of *Paramecium*. One may therefore conclude that the *chemical changes underlying the pathological conditions in tartrate nephritis are responsible for the effect noted upon Paramecium. Conversely, Paramecium may be relied upon to give evidence of such chemical alterations under the conditions of our experiment.*

#### CONCLUSIONS.

*Paramecium* fails to indicate any essential difference in its division rate when subjected to the influence of extracts prepared from the separate kidneys of one rabbit or from kidneys of different individuals. Kidney extracts made from a starving rabbit behave in a manner identical with normal kidney extracts of well-fed animals.

The division rate of *Paramecium* is markedly depressed when placed in kidney extracts of rabbits with tartrate nephritis.

It has been demonstrated that this depressant influence cannot be associated primarily with tartrate which has accumulated in the kidneys, since quantities which could be present according to chemical tests would produce the opposite effect, namely slight stimulation of the division rate. Moreover, the depressant action of kidney extracts prepared from animals receiving large doses of tartrate is no greater than with extracts of nephritic kidneys of animals that had been given small doses of tartrate.

From these facts it is apparent that the pathological change—hence, presumably chemical alteration—in the renal tissue itself is responsible for the action observed upon the division rate of *Paramecium*.

*Paramecium* therefore may be regarded as a biological indicator of chemical change; and it is proposed to employ this biological method for the detection of chemical change under a variety of normal and pathological conditions.

## PROTOZOAN PROTOPLASM AS AN INDICATOR OF PATHOLOGICAL CHANGES.

### II. IN CARCINOMA.

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The detrimental influence of cancer growth upon the organism in general indicates that in the development of neoplasms abnormal metabolic conditions obtain. It is not improbable that under such circumstances there arise products from the altered metabolism which account in a measure for the observed symptoms. The extensive literature upon the subject fails to reveal positive evidence of the existence of such substances which may be held responsible for the clinical picture presented, although experiments on the influence of various products of normal metabolic activity on protozoa, seedlings, etc., have been carried out.<sup>1</sup>

The importance of determining the presence of substances which may exert a detrimental action upon metabolic processes is obvious. We believe that the method outlined by us in a previous paper<sup>2</sup> for indicating chemical change is sufficiently reliable to demonstrate whether substances inimical to protoplasm are present in cancerous growths. We have therefore applied this method to the problem under discussion, employing primary carcinoma of the human breast as a characteristic type of abnormal growth.<sup>3</sup>

#### *Methods.*

In the cases here recorded the entire breast had been removed. Immediately after the operation extracts of uninvolved mammary tissue and carcinomatous tissue were prepared in exact accordance

<sup>1</sup> Calkins, Bullock and Rohdenburg: *Journ. of Inf. Dis.*, x, p. 421, 1912; Rosenbloom: *Biochem. Bull.*, ii, p. 229, 1912.

<sup>2</sup> Woodruff and Underhill: this *Journal*, xv, p. 385, 1913.

<sup>3</sup> We are greatly indebted to Dr. William F. Verdi and Dr. Otto G. Ramsay for placing the tissues employed at our disposal.

with the directions previously outlined.<sup>4</sup> Extreme precautions were taken to exclude all possible sources of contamination of the tissues. The rate of division of paramecia bred in the extract of the carcinoma has been compared with that in the extract of normal tissue of the same breast. The method of conducting the paramecia cultures is identical with that outlined in the preceding paper.<sup>5</sup>

*The effect of carcinoma extracts upon the division rate of Paramecium.*

In the preliminary experiments (Table I, Case 1) and Table II, Case 2) it is demonstrated that in the dilutions employed carcinoma extracts have little or no influence on the division rate of paramecia when compared with the growth of these organisms upon normal breast-tissue extracts. When, however, the original, undiluted extracts are employed an entirely different result is obtained (see Table III, Cases 1 and 2). Under these experimental conditions carcinoma extracts exert a primary depressant influence upon the division rate of *Paramecium* which invariably results in death within a period of from two to three days.

In Table IV, Case 3, are given the details of an experiment carried through a period of ten days. Inspection of these data will reveal several points of significance. In the first place it is evident that the original undiluted carcinoma extract has a marked depressant influence upon the rate of division of *Paramecium*, which is a direct confirmation of the results obtained in Cases 1 and 2. On the other hand, unlike those of Cases 1 and 2, the extract of Case 3 did not cause death in a single line of the culture. When the original extracts were diluted even very greatly, for instance 1:24, an effect was obtained just the opposite to that of the undiluted extract—a significant stimulation resulting.

From the figures given below (Table V) it is apparent that *Paramecium* is capable of distinguishing between the different concentrations of the same solution whether derived from normal or pathological tissue, thus again<sup>6</sup> indicating the sensitiveness of

<sup>4</sup> Woodruff and Underhill: *loc. cit.*

<sup>5</sup> *Ibid.*

<sup>6</sup> Woodruff and Bunzel: *Amer. Journ. of Physiol.*, xxv, p. 190, 1909.

TABLE I.

*The effects upon the division rate of Paramecium of dilute solutions of carcinoma extracts.*

## Case 1.

SUBSTANCE TESTED	CULTURE	NO. OF DIVISIONS AFTER DAYS				
		1	2	3	4	5
Normal tissue Dilution = 1:3	A.....	3	17	28	41	49
	B.....	2	14	24	38	45
	Total.....	5	31	52	79	94
Carcinoma Dilution = 1:3	A.....	4	16	24	38	47
	B.....	4	13	21	33	41
	Total.....	8	29	45	71	88
Normal tissue Dilution = 1:6	A.....	3	15	24	39	44
	B.....	2	15	25	38	45
	Total.....	5	30	49	77	89
Carcinoma Dilution = 1:6	A.....	6	19	26	34	41
	B.....	5	19	27	38	45
	Total.....	11	38	53	72	86
Normal tissue Dilution = 1:12	A.....	3	14	21	33	40
	B.....	4	14	22	35	39
	Total.....	7	28	43	68	79
Carcinoma Dilution = 1:12	A.....	4	14	23	35	41
	B.....	4	15	22	30	37
	Total.....	8	29	45	65	78
Normal tissue..... Dilution = 1:24	A.....	4	13	16	31	35
	B.....	2	12	18	33	37
	Total.....	6	25	34	64	72
Carcinoma Dilution = 1:24	A.....	5	10	16	26	29
	B.....	5	14	22	31	37
	Total.....	10	24	38	57	66

## Carcinoma

TABLE II.

*The effects upon the division rate of Paramecium of dilute solutions of carcinoma extracts.*

## Case 2.

SUBSTANCE TESTED	CULTURE	NO. OF DIVISIONS AFTER DAYS			
		1	2	3	4
Normal tissue Dilution = 1:3	A.....	8	16	29	42
	B.....	8	15	28	40
		—	—	—	—
	Total.....	16	31	57	82
Carcinoma Dilution = 1:3	A.....	7	15	29	39
	B.....	8	16	28	39
		—	—	—	—
	Total.....	15	31	57	78
Normal tissue Dilution = 1:6	A.....	8	17	29	41
	B.....	6	12	27	40
		—	—	—	—
	Total.....	14	29	56	81
Carcinoma Dilution = 1:6	A.....	8	16	29	42
	B.....	6	15	29	41
		—	—	—	—
	Total.....	14	31	58	83
Normal tissue Dilution = 1:12	A.....	8	14	29	36
	B.....	8	14	26	35
		—	—	—	—
	Total.....	16	28	55	71
Carcinoma Dilution = 1:12	A.....	8	13	22	33
	B.....	8	16	23	36
		—	—	—	—
	Total.....	16	29	45	69



TABLE III.

*The effects upon the division rate of Paramecium of strong and weak extracts of carcinoma.*

SUBSTANCE TESTED	CULTURE	CASE 1		CASE 2	
		NO. OF DIVISIONS AFTER DAYS			
		1	2	1	2
Normal tissue Undiluted	A.....	5	15	7	22
	B.....	5	15	8	22
		—	—	—	—
	Total.....	10	30	15	44
Carcinoma Undiluted	A.....	0	0	1	4
	B.....	0	0	2	6
		—	—	—	—
	Total.....	0	all dead	3	10 all dead
Normal tissue Dilution = 1:3	A.....	11	25	9	21
	B.....	10	24	10	21
		—	—	—	—
	Total.....	21	49	19	43
Carcinoma Dilution = 1:3	A.....	8	24	10	21
	B.....	8	23	9	20
		—	—	—	—
	Total.....	16	47	19	41
Normal tissue Dilution = 1:6	A.....	8	21	8	18
	B.....	9	21	8	18
		—	—	—	—
	Total.....	17	42	16	36
Carcinoma Dilution = 1:6	A.....	10	21	10	22
	B.....	8	21	8	20
		—	—	—	—
	Total.....	18	42	18	42

## Carcinoma

TABLE IV.

*The effects on the division rate of Paramecium of strong and weak extracts of carcinoma.*

*Case 3.*

SUBSTANCE TESTED	CULTURE	NO. OF DIVISIONS AFTER DATE									
		1	2	3	4	5	6	7	8	9	10
Normal tissue Undiluted	A.....	5	17	30	38	49	58	71	81	89	103
	B.....	8	22	33	42	54	62	75	85	92	110
	Total.....	13	39	63	80	103	120	146	166	181	213
Carcinoma Undiluted	A.....	4	14	22	27	30	34	36	39	43	45
	B.....	5	14	22	27	32	35	37	39	42	45
	Total.....	9	28	44	54	62	69	73	78	85	90
Normal tissue Dilution = 1:3	A.....	6	18	29	36	47	56	69	76	84	102
	B.....	5	16	28	35	44	52	63	70	78	96
	Total.....	11	34	57	71	91	108	132	146	162	198
Carcinoma Dilution = 1:3	A.....	6	20	33	41	52	61	75	83	91	109
	B.....	8	22	35	40	52	62	76	84	92	110
	Total.....	14	42	68	81	104	123	151	167	183	219
Normal tissue Dilution = 1:6	A.....	4	15	25	27	35	42	51	60	64	78
	B.....	5	15	23	28	34	39	46	55	58	63
	Total.....	9	30	48	55	69	81	97	115	122	140
Carcinoma Dilution = 1:6	A.....	6	18	29	34	41	48	56	63	69	83
	B.....	4	19	30	35	42	49	58	68	76	95
	Total.....	10	37	59	69	83	97	114	131	145	178
Normal tissue Dilution = 1:12	A.....	5	13	17	21	25	29	36	42	48	59
	B.....	5	13	17	21	25	30	33	37	41	50
	Total.....	10	26	34	42	50	59	69	79	89	109
Carcinoma Dilution = 1:12	A.....	8	18	29	37	44	49	59	67	74	89
	B.....	6	15	27	31	40	46	51	59	66	76
	Total.....	14	33	56	68	84	95	110	126	140	165
Normal tissue Dilution = 1:24	A.....	3	9	13	14	17	18	20	22	24	26
	B.....	1	8	12	13	19	21	26	28	31	36
	Total.....	4	17	25	27	36	39	46	50	55	62
Carcinoma Dilution = 1:24	A.....	5	14	22	26	29	34	43	49	54	68
	B.....	6	17	25	28	31	35	40	45	50	52
	Total.....	11	31	47	54	60	69	83	94	104	120

this organism to quantitative chemical changes in its environment and therefore its value as an indicator of quantitative changes as well as of alterations presumably of a qualitative character.

The depressing influence of strong solutions of cancer extracts again finds corroboration in the data of Cases 4 and 5 contained in Tables VI and VII. Unlike those of Case 3, however, the weaker dilutions fail to reveal any stimulating influence upon the division rate of *Paramecium*. The detection of quantitative differences in the two solutions under discussion is again in evidence (see Table VIII) although the differences are not so sharply defined as in Case 3. In Cases 4 and 5, however, the strongest dilution of both solutions usually shows a tendency to inhibit the division rate of *Paramecium* when compared with extracts that have been diluted 1:3.

TABLE V.

*Case 3.*

DILUTION	DIVISIONS IN NORMAL EXTRACT		DIVISIONS IN CARCINOMATOUS EXTRACT	
	5 days	10 days	5 days	10 days
Undiluted	103	213	62	90
1:3	91	198	104	219
1:6	69	140	83	178
1:12	50	109	84	165
1:24	36	62	60	116

From a survey of the results of the five cases of carcinoma of the breast presented it is evident that in each instance the original undiluted extract of the abnormal tissue exerts a very pronounced depressant influence upon the division rate of *Paramecium* when bred under the conditions of our experiments. In certain instances this action may be so significant as to lead to death of the organisms within a comparatively short time. Weaker solutions of the abnormal extract may or may not show a stimulating action when tested toward *Paramecium*.

TABLE VI.

*The effects upon the division rate of Paramecium of strong and weak extracts of carcinoma.*

*Case 4.*

SUBSTANCE TESTED	CULTURE	NO. OF DIVISIONS AFTER DAYS				
		1	2	3	4	5
Normal tissue Undiluted	A.....	9	20	32	48	56
	B.....	6	16	28	40	49
	Total.....	15	36	60	88	105
Carcinoma Undiluted	A.....	4	13	20	29	35
	B.....	4	13	22	32	36
	Total.....	8	26	42	61	71
Normal tissue Dilution = 1:3	A.....	9	20	34	50	57
	B.....	8	19	32	49	56
	Total.....	17	39	66	99	113
Carcinoma Dilution = 1:3	A.....	7	19	31	47	53
	B.....	9	18	32	49	54
	Total.....	16	37	63	96	107
Normal tissue Dilution = 1:6	A.....	8	19	32	47	54
	B.....	8	16	28	44	52
	Total.....	16	35	60	91	106
Carcinoma Dilution = 1:6	A.....	9	17	29	45	51
	B.....	8	18	31	47	52
	Total.....	17	35	60	92	103
Normal tissue Dilution = 1:12	A.....	11	17	28	42	48
	B.....	8	16	26	40	46
	Total.....	19	33	54	82	94
Carcinoma Dilution = 1:12	A.....	9	14	27	41	48
	B.....	10	16	29	44	50
	Total.....	19	33	56	85	98
Normal tissue Dilution = 1:24	A.....	7	13	22	36	42
	B.....	8	13	23	35	42
	Total.....	15	26	45	71	84
Carcinoma Dilution = 1:24	A.....	8	15	25	36	40
	B.....	7	12	24	36	40
	Total.....	15	27	49	72	80

TABLE VII.

*The effects upon the division rate of Paramecium of strong and weak extracts of carcinoma.*

*Case 5.*

SUBSTANCE TESTED	CULTURE	NO. OF DIVISIONS AFTER DAYS				
		1	2	3	4	5
Normal tissue Undiluted	A.....	8	18	32	44	55
	B.....	5	17	30	40	50
	Total.....	13	35	62	84	105
Carcinoma Undiluted	A.....	2	5	7	12	15
	B.....	2	6	12	17	20
	Total.....	4	11	19	29	35
Normal tissue Dilution = 1:3	A.....	7	19	29	38	48
	B.....	6	18	30	39	48
	Total.....	13	37	59	77	96
Carcinoma Dilution = 1:3	A.....	7	18	31	39	49
	B.....	8	20	32	40	52
	Total.....	15	38	63	79	101
Normal tissue Dilution = 1:6	A.....	6	17	30	38	49
	B.....	6	20	30	39	48
	Total.....	12	37	60	77	97
Carcinoma Dilution = 1:6	A.....	5	16	29	38	47
	B.....	7	18	30	39	51
	Total.....	12	34	59	77	98
Normal tissue Dilution = 1:12	A.....	7	16	28	36	45
	B.....	7	17	29	35	44
	Total.....	14	33	57	71	89
Carcinoma Dilution = 1:12	A.....	6	14	26	33	42
	B.....	6	15	27	34	42
	Total.....	12	29	53	67	84
Normal tissue Dilution = 1:24	A.....	6	14	22	28	33
	B.....	5	13	21	29	34
	Total.....	11	27	43	57	67
Carcinoma Dilution = 1:24	A.....	6	13	24	29	38
	B.....	6	13	24	31	37
	Total.....	12	26	48	60	75

*The influence upon Paramecium of extracts of normal and carcinomatous breast tissue of similar concentration.*

In view of the sensitiveness of paramecia toward quantitative differences in the same solution it is possible that the influence of cancerous tissue extract may be accounted for entirely by assuming that this extract was merely quantitatively different from the normal extract. To test this hypothesis analyses were made of the extracts prepared from normal breast tissue and from the carcinoma in Cases 3, 4 and 5—total nitrogen, solids and ash being estimated (see Table IX).

TABLE VIII.

## Case 4.

DILUTION	NORMAL	ABNORMAL
Undiluted	105 divisions	71 divisions
1:3	113 divisions	107 divisions
1:6	106 divisions	103 divisions
1:12	94 divisions	98 divisions
1:24	84 divisions	80 divisions

## Case 5.

Undiluted	105 divisions	35 divisions
1:3	96 divisions	101 divisions
1:6	97 divisions	98 divisions
1:12	89 divisions	84 divisions
1:24	67 divisions	75 divisions

TABLE IX.

## Composition of tissue extracts.

CASE	TISSUE EXTRACT	TOTAL N	SOLIDS	ASH
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
3	Normal.....	0.038	0.36	0.045
	Abnormal.....	0.060	0.63	0.073
4	Normal.....	0.050	0.43	0.047
	Abnormal.....	0.033	0.36	0.032
5	Normal.....	0.051	0.40	0.045
	Abnormal.....	0.039	0.34	0.030

These figures demonstrate that in Case 3 the greatest quantitative difference is to be found, the abnormal tissue extract being noticeably stronger than the normal breast extract. On the other hand the concentration of the normal extract in Cases 4 and 5 is considerably higher than the corresponding carcinoma extract. It is therefore apparent that mere quantitative differences in composition cannot be the sole cause for the effect upon *Paramecium* observed, since in the three cases the action of the original undiluted extracts was in the same direction, namely, marked depression of the division rate. In order, however, to further test this point the following experiments were carried out. Cases 3 and 4 were selected as typical examples—the abnormal extract in Case 3 being relatively much stronger than the normal extract; the normal extracts of Cases 4 and 5 being stronger than the cancerous extracts. Owing to lack of material in Case 5 it was deemed sufficient to take Case 4 as typical inasmuch as the two normal extracts of Cases 4 and 5 and the two extracts of abnormal tissue were almost identical in composition. Having selected these two cases as typical of the two tendencies in concentration of the substances determined, the stronger solution in each case was diluted with water in such a manner as to make it approximately equal in concentration to the weaker extract. The two solutions of each case, now nearly alike in concentration, were further diluted with water as in previous experiments and the resulting solutions were employed as culture media for *paramecia* with the results outlined in Tables X and XI.

It is at once apparent from these figures that the dilution of the stronger solution in each case did not affect the original characteristic action of these extracts upon the division rate of *Paramecium*. In other words, even when the two solutions in each case were made approximately alike in concentration as determined by the total solids, nitrogen and ash content, the marked depressant effect of the strong solutions may be observed upon the division rate of *Paramecium*. Likewise the general characteristics of the solutions are preserved. The stimulating action of the weaker solutions of Case 3 observed previously is again evident, whereas in Case 4 such an influence is inconstant—again in entire agreement with the results obtained with Case 4 outlined above.

## Carcinoma

TABLE X.

*The effects upon the division rate of Paramecium of extracts of carcinoma made similar in concentration to extracts of normal mammary tissue.*

*Case 3.*

SUBSTANCE TESTED	CULTURE	NO. OF DIVISIONS AFTER DAYS				
		1	2	3	4	5
Normal tissue Undiluted	A.....	8	20	34	43	53
	B.....	8	19	32	42	50
	Total.....	16	39	66	85	103
Carcinoma Undiluted	A.....	0	5	14	21	26
	B.....	0	5	13	18	24
	Total.....	0	10	27	39	50
Normal tissue Dilution = 1:3	A.....	7	15	26	32	39
	B.....	7	14	26	34	41
	Total.....	14	29	52	66	80
Carcinoma Dilution = 1:3	A.....	7	11	22	28	35
	B.....	6	13	21	29	35
	Total.....	13	24	43	57	70
Normal tissue Dilution = 1:6	A.....	8	12	19	23	28
	B.....	8	13	21	23	27
	Total.....	16	25	40	46	55
Carcinoma Dilution = 1:6	A.....	7	15	24	30	36
	B.....	6	14	23	29	33
	Total.....	13	29	47	59	69
Normal tissue Dilution = 1:12	A.....	5	11	16	21	22
	B.....	5	6	11	16	17
	Total.....	10	17	27	37	39
Carcinoma Dilution = 1:12	A.....	6	12	22	28	34
	B.....	6	12	23	29	33
	Total.....	12	24	45	57	67
Normal tissue Dilution = 1:24	A.....	5	9	16	18	18
	B.....	4	9	14	18	21
	Total.....	9	18	30	36	39
Carcinoma Dilution = 1:24	A.....	4	10	19	25	28
	B.....	4	10	13	19	21
	Total.....	8	20	32	44	49



TABLE XI.

*The effects upon the division rate of Paramecium of extracts of carcinoma made similar in concentration to extracts of normal mammary tissue.*

*Case 4.*

SUBSTANCE TESTED	* CULTURE	NO. OF DIVISIONS AFTER DAYS				
		1	2	3	4	5
Normal tissue Undiluted	A.....	8	19	31	45	59
	B.....	8	19	31	45	57
	Total.....	16	38	62	90	116
Carcinoma Undiluted	A.....	1	3	4	4	4
	B.....	1	4	7	10	11
	Total.....	2	7	11	14	15
Normal tissue Dilution = 1:3	A.....	8	14	28	40	52
	B.....	9	19	31	44	59
	Total.....	17	33	59	84	111
Carcinoma Dilution = 1:3	A.....	10	19	28	40	54
	B.....	10	18	30	41	53
	Total.....	20	37	58	81	107
Normal tissue Dilution = 1:6	A.....	10	14	24	37	47
	B.....	8	12	22	34	45
	Total.....	18	26	46	71	92
Carcinoma Dilution = 1:6	A.....	9	16	26	38	49
	B.....	9	16	28	40	52
	Total.....	18	32	54	78	101
Normal tissue Dilution = 1:12	A.....	7	14	22	34	44
	B.....	5	11	19	31	39
	Total.....	12	25	41	65	83
Carcinoma Dilution = 1:12	A.....	5	9	18	28	37
	B.....	7	11	18	30	40
	Total.....	12	20	36	58	77
Normal tissue Dilution = 1:24	A.....	6	9	16	24	32
	B.....	6	8	16	24	33
	Total.....	12	17	32	48	65
Carcinoma Dilution = 1:24	A.....	4	8	17	25	35
	B.....	5	10	17	25	33
	Total.....	9	18	34	50	68

The effect of carcinoma extracts upon the division rate of *Paramecium* may be due to the absence or to a deficiency in these solutions of substances essential for the life processes of *Paramecium*, or to the presence in these extracts of substances inimical to protoplasm. If the latter hypothesis is correct it is possible, in view of the probable greater concentration of these substances in the neoplasm, that their absorption may contribute to the production of the symptoms characteristic of the development of cancerous growths. Further work on cancerous tissues is in progress tending toward the solution of some of the obvious problems suggested by our results.

#### CONCLUSIONS.

*In certain concentrations extracts of carcinoma of the breast show a very pronounced depressant influence upon the division rate of Paramecium when compared to that obtained with Paramecium bred in normal mammary tissue extract. In some instances the depressant influence may be so profound as to lead to the death of the paramecia within two or three days.*

Weaker dilutions of the abnormal tissue extracts may show a stimulating action upon *Paramecium*.

It has been demonstrated that the difference in concentration which may exist between the normal and abnormal breast tissue extracts cannot be held responsible for the detrimental action of the abnormal extract upon *Paramecium*, for when the concentrations of the two extracts under discussion are made as nearly equal as possible the original type of action still is observed.

## ON THE FORMATION OF HYDROCYANIC ACID FROM PROTEINS.

By H. W. EMERSON, H. P. CADY AND E. H. S. BAILEY.

*(From the Department of Chemistry, University of Kansas.)*

(Received for publication, June 28, 1913.)

The question arose during the progress of a recent criminal trial whether decomposing proteins could, under any condition, liberate hydrocyanic acid. A search of the literature available failed to reveal any instances recorded. A great many experiments were therefore started in order to determine the question.

The method of testing for hydrocyanic acid was the Schönbein test with the modifications as suggested by Dr. Walter S. Haines, which are as follows:

A strip of fresh ash-free Swedish filter paper, about 8 cm. long and 0.5 cm. wide, is dipped about one-third of its length into a freshly prepared 10 per cent tincture of guaiac, then withdrawn and held upright in the air for a few moments until some of the alcohol has evaporated; then a drop of aqueous copper sulphate, one to one-thousand (1-1000) was placed on the tip of the filter paper so that it moistens not more than half of that which has been wet with the tincture of guaiac. The paper thus prepared is suspended above the solution to be tested for hydrocyanic acid for one minute and if hydrocyanic acid is present, even in dilutions as great as 1 part to 3,000,000, it will color distinctly blue that part of the paper only which has been wet with copper sulphate. If a volatile oxidizing agent is present, it will color blue, not only the tip which has been moistened with both copper sulphate and tincture of guaiac, but also that part which has been moistened with the tincture of guaiac.

The test as applied in this manner affords at the same time then a good way of distinguishing between hydrocyanic acid and volatile oxidizing substances. Ammonia, when it is present, will turn the entire paper a bluish green if dilute and a yellowish brown color if more concentrated. The presence of ammonia can be prevented by keeping the solution slightly acid. Hydrogen sulphide, if it is present, interferes with the test and if a guaiac-copper sulphate paper which has been first turned blue by hydrocyanic acid fumes is placed in hydrogen sulphide, the hydrogen sulphide will bleach out the blue color quite rapidly.

## 416 Hydrocyanic Acid Formation from Proteins

Eggs were used in the first experiments: They were broken and the whites separated from the yolk and distributed into wide mouthed 500 cc. flasks so that each flask contained either the whites or the yolks of two eggs. The flasks were loosely stoppered and put in a warm place and tested each day by the Schönbein test for hydrocyanic acid.

Seventy-four experiments were started in nine different series and in eight experiments from six different series, after intervals varying from four days to fourteen days, the egg substance evolved hydrocyanic acid. In one case the egg substance continued to give off hydrocyanic acid for thirty-six days after which time it had all been used up inoculating other protein media. The eggs which gave the test best remained acid to litmus and phenolphthalein, but, at no time, were acid to dimethyl amido-azobenzol. They did not become putrid like those which decomposed without giving off hydrocyanic acid. We have good reasons for believing that a number of the eggs which in decomposing became putrid, also gave off hydrocyanic acid, but the hydrocyanic acid escaped detection as the hydrogen sulphide interfered with the test used. In a number of such cases, we obtained crystals which looked like silver cyanide although the silver nitrate was darkened by the hydrogen sulphide, but we were not able to get positive results with the Prussian blue test.

The yolks developed hydrocyanic acid in six cases and the whites in two cases and inoculation experiments showed egg yolk to be a more favorable medium than egg white.

That it was hydrocyanic acid that was given off was confirmed by obtaining the characteristic silver cyanide crystals, then decomposing these crystals with sodium hydroxide, ferrous sulphate and ferric chloride and subsequent acidulating with hydrochloric acid and obtaining the Prussian blue color. In some cases, the odor of hydrocyanic acid was easily detected when the stopper to the flask was first removed. That the reaction was not due to sulphocyanic acid was shown by dipping some filter paper in ferric chloride solution and suspending this over the liquid in the flask and the paper did not turn red as it does in the presence of sulphocyanic acid.

That the decomposition of this protein was due to some micro-organism was indicated by the following facts. A great many

inoculations were made from solutions that were giving off hydrocyanic acid abundantly, into fresh media and in every case, after incubation for twenty-four hours, they gave good tests for hydrocyanic acid. The organism was killed by the presence of free mineral acid, and also was destroyed by heating.

Egg culture 41, was, therefore, given to Messrs. Clawson and Young of the Department of Bacteriology to determine for us the organism which, in living on protein media, gave off hydrocyanic acid. They determined it to be *Bacillus pyocyaneus*.<sup>1</sup>

The organism gives off hydrocyanic acid in larger quantities when grown on yolk than when grown on white. It is a good hydrocyanic acid producer when grown on gelatin, which it liquefies. It will produce hydrocyanic acid when grown on milk, but not abundantly, and when grown on liver tissue.

The organism grows best, or rather gives off hydrocyanic acid more rapidly when grown in a medium that is slightly acid to litmus and phenolphthalein. Repeated trials showed that the organism does not develop hydrocyanic acid at all when placed in eggs to which just sufficient hydrochloric acid has been added to give a test for free mineral acid.

When there is added to a culture, that is giving a good test for hydrocyanic acid, sufficient 2 per cent hydrochloric acid to just give a test for free mineral acid and sufficient time allowed for the hydrocyanic acid, which had been developed before, to disappear, the culture will no longer develop hydrocyanic acid. And if new media is now inoculated with this solution, it will not give off hydrocyanic acid which indicates that the presence of free mineral acid kills the organism.

The experiments are being continued and will be extended to other organisms.

#### CONCLUSIONS.

1. Certain microorganisms living on protein media evolve hydrocyanic acid.
2. These organisms liberate hydrocyanic best when living on a protein media slightly acid to litmus and phenolphthalein.
3. They do not liberate hydrocyanic acid in media containing free mineral acid.

<sup>1</sup> See this *Journal*, xv, p. 419, 1913.



## PRELIMINARY REPORT ON THE PRODUCTION OF HYDROCYANIC ACID BY BACTERIA.

BY B. J. CLAWSON AND C. C. YOUNG.

(From the Department of Bacteriology, University of Kansas.)

(Received for publication, June 28, 1913.)

Egg culture 41 which had produced HCN spontaneously, and which is mentioned in the paper by Emerson, Cady, and Bailey<sup>1</sup> was turned over to us by Dr. Cady for investigation to determine the organism, if any, which caused the generation of hydrocyanic acid. The modified Schönbein test was used throughout this work to detect the presence of hydrocyanic acid gas.

Stained smears were made of the original material in which an almost pure culture of a short rod was seen. Transfers of this material to sterilized whole egg developed hydrocyanic acid in twenty-four hours. From these tubes, the material was plated on agar and grown at room temperature. After forty-eight hours, colonies appeared which produced a blue-green pigment which was distributed through the agar. Transfers to gelatin showed rapid liquefaction at room temperature. After forty-eight hours, the presence of HCN gas was revealed by the Schönbein test and confirmed by the Prussian blue reaction.

The principle characteristics of the organism are as follows: short rod; no spores; slightly pointed at the ends; decidedly motile; Gram negative; liquefies gelatin rapidly at 22°; brown on potato; peptonizes milk which becomes slightly alkaline; reduces nitrates to nitrites and ammonia; produces indol; no fermentation or production of gas in lactose, dextrose, saccharose, raffinose, salicin, inulin, mannite, or dulcitate broth media. Does not reduce neutral red; forms heavy pellicle on broth, no fluorescent pigment containing pyocyanin on agar. It does not grow in media which are even slightly acid to sodium sulphionate.

*Journal*, xv, p. 415, 1913.

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From the above we conclude that the organism is *Bacillus pyocyaneus*. No attempt was made in this work to distinguish between *Bacillus pyocyaneus* and *Bacillus fluorescens* as both have the HCN-producing power.

The organism was grown on 50 grams of gelatin in an Erlenmeyer flask at room temperature. After growing for twenty-four hours and testing for HCN production, the flask was connected with a U tube containing 2 cc. of a 10 per cent solution of silver nitrate. The air that filtered through the cotton plug was drawn slowly through the flask and U tube for seventy-two hours. The nitrate and precipitate were then transferred to a small distilling flask and strong HCl added. Five cubic centimeters were distilled off into a receiver buried in freezing mixture. Of this distillate 0.2 cc. killed a chick in less than thirty seconds; 0.4 cc. given to a three-weeks' old kitten, killed it almost instantly.

The power which the organism has of producing HCN, is apparently not due to an extracellular enzyme. The organism was grown in gelatin for seventy-two hours at 37°, and had been giving off HCN for forty-eight hours. After filtering through a Berkefeld filter, some of the filtrate was planted again into gelatin and incubated, but gave negative results for HCN.

Hydrocyanic acid gas is apparently produced only under aerobic conditions, which led to the belief that the reaction in which HCN was produced was due to oxidation of the proteins. This was subsequently shown to be true. While good tests were always obtained from cultures grown at room temperature (22°), as a rule cultures grown at 37° gave a stronger test for HCN. Different media were used to determine whether or not the ability to produce HCN would be shown in them. Positive tests were obtained at 37° from gelatin, broth, milk, agar, Dunham's peptone solution, cotton seed meal, and egg, in which several different proteins are involved. The growth of the organism on egg increased the HCN production decidedly. This was true for all strains of *pyocyaneus* tried. This increase did not diminish when it was subsequently grown on other protein media.

Several other strains of *B. pyocyaneus* were tested for HCN production, all of which returned positive results. They were from the following sources: (1) University of Chicago stock culture; (2) egg which had been frozen two and one-half years; (3) soil; (4) Kaw river.



The stock culture of *B. pyocyaneus* from the University of Chicago, when first planted into gelatin, gave a very weak test for HCN; but after twenty-four to forty-eight hours' growth in egg, it became a strong producer of hydrocyanic acid. It would seem that the organism, living as a saprophyte, is better prepared to decompose protein substances with the production of HCN, than when living as a parasite. A rabbit was injected intraperitoneally with several cubic centimeters of a twenty-four-hour broth culture of the University of Chicago strain. After three days the rabbit died. An autopsy showed general peritonitis. Transfers made from the heart's blood gave a pure culture of *B. pyocyaneus*. This was grown in gelatin, but gave only a faint test for HCN which indicates that the organism living in an animal is reduced in its power to produce HCN. It was then grown for twenty-four hours on sterilized egg, when the test for hydrocyanic acid was one of the strongest obtained. Subsequent transfers from the egg to gelatin showed no diminution of the production of HCN. An attempt to grow the organism on Jordan's synthetic media<sup>2</sup> was unsuccessful.

*B. Pyocyaneus* was not the only organism found which produced HCN from proteins. Miss Myrtle Greenfield, bacteriologist for the State Water Survey, isolated an organism from soil, the name of which has not been determined. It was a strong HCN producer. The characteristics of the organism are as follows: long rod; rounded ends; no spores; very motile; takes all ordinary stains; Gram negative; abundant growth on all ordinary media; growth on agar glistening; orange pigment diffusing rapidly through agar, gelatin, potato and milk; deep colonies in agar fusiform; liquefies gelatin rapidly; gelatin stab infundibuliform. On broth and milk, a delicate pellicle is formed, and the media are colored from the surface down. Milk is gradually peptonized. Litmus is reduced. All media are made slightly alkaline. There is no diastatic action on potato starch. A slight amount of nitrite is formed. The organism grows best at 20° and is aerobic. No acid or gas is formed in dextrose, lactose, saccharose or glycerin broth.

<sup>2</sup> *Journ. of Exp. Med.*, iv, p. 629, 1899; Jordan: *Botanical Gazette*, xxvii, p. 9, 1899.

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A culture of *Bacillus violaceus* from the American Museum of Natural History, was also found to produce HCN in gelatin and egg. It is very possible that other chromogens may have this same property.

A number of liquefying organisms were tested and indications of the formation of hydrocyanic acid were obtained although the tests were influenced by ammonia and sulphuretted hydrogen. They will be examined more carefully.

There has been much work done on the production of HCN from grain, beans, linseed meal, germinating *Sorghum vulgares*, and other protein-containing substances. In most cases, the production of HCN is attributed to an enzyme. Apparently all of the workers were using non-sterile material, which could have been easily contaminated by an HCN-producing organism.<sup>3</sup>

The authors are continuing the investigation with the intention of publishing more complete information concerning the decomposition of protein substances with the production of HCN due to the microorganisms mentioned in the body of the paper.

<sup>3</sup> L. Guignard: HCN in Beans, *Recueil actes off. et doc. interessant hyg. pub., travaux Conseil Supérieur Hyg. Pub. France*, 1909; S. J. M. Auld, Jr.: HCN in Linseed Cake and Other Feeding Stuff, South East Agricultural College, Wye, England, No. 20, pp. 289-320, 1911; C. Ravenna (Univ. of Bologna): HCN from Seeds of Sorghum, *Atti R. accad. dei Lincei*, xix, II, pp. 356-361; HCN from Seeds of Sorghum, *Gaz. chim. ital.*, xli, II, pp. 74-81; C. Ravenna and M. Zamorani (Lab. Agric. Chem.): Physiological Function of HCN in Plants, *Chem. Zentralbl.*, i, p. 113, 1910; M. J. Offner: HCN in Fungi, *Bull. soc. mycol. de France*, xxvii, pp. 342-5; S. J. M. Auld, Jr.: Formation of HCN in Linseed Cake and other Food Stuffs, London Board of Agriculture, No. 6, pp. 446-460; No. 8, pp. 657-660, 1912; C. D. Londer: Formation of HCN in Linseed Cake and other Food Stuffs, London Board of Agriculture, No. 11, pp. 904-907, 1911.

## CONTRIBUTIONS TO THE CHEMICAL DIFFERENTIATION OF THE CENTRAL NERVOUS SYSTEM.

### III. THE CHEMICAL DIFFERENTIATION OF THE BRAIN OF THE ALBINO RAT DURING GROWTH.

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(Received for publication, July 1, 1913.)

#### INTRODUCTORY STATEMENT.<sup>1</sup>

The transformations which occur in the brain during growth offer a particularly enticing field for the study of chemical differentiation, not alone because of the very great interest attaching to the solution of the problem of the chemical basis of its functions, but because its structural differentiation during growth is very marked. On the one hand there is the formation of a large amount of new material composing the medullary sheath of the nerve fibers, and, on the other hand, the appearance of a quantity of a peculiar supporting tissue, the neuroglia. The chemical changes during growth should, therefore, be very marked; and it is of interest to discover how far our chemical methods enable us to follow such obvious structural modifications.

<sup>1</sup> Waldemar Koch died at Chicago February 1, 1912. As Associate in Biological Chemistry at the Wistar Institute of Anatomy and Biology, he spent the autumn of 1910 and of 1911 in Philadelphia working mainly on matters connected with this research. This paper has been prepared in considerable part from results of analyses made by me under the direction of my brother, and from a manuscript written by him. Many additional analyses, which he had planned, have been made and incorporated into the series. The interpretations of the results have been left to a large extent, in his words. I have been assisted in its preparation for publication by Professor A. P. Mathews and Dr. H. H. Donaldson, the aid of both of whom I gratefully acknowledge.—M. L. KOCH.

The selection of chemical methods for such a study was largely guided by the principle now coming to be generally accepted, namely, that in living matter we are not dealing with an aggregation of more or less similar, highly organized and necessarily complex molecules (*Riesenmolekül* of Pflüger), but rather, with a more or less heterogeneous substratum in which dissimilar and not necessarily highly complex molecules, or their dissociated particles, are engaged in a series of correlated chemical reactions. The larger aggregates may be conceived as either not taking part directly in chemical activity, or as helping in the control and localization of the chemical reactions, just as in a photographic dry plate, the presence of the gelatin makes possible a high degree of localization of the photo-chemical reaction. We aimed, therefore, to stop the chemical activities at definite given stages during the growth period and then to observe the differences which could be demonstrated. From such data we then drew conclusions as to the nature of the transformations which had occurred in the interval.

The methods of collecting the material were devised with this end in view, namely, to stop all chemical activity as rapidly and completely as possible. The sources of error due to post mortem changes then became constant, and we are in reality following a principle that has long been in use in histological studies. For the preserving agent, alcohol was selected, as it is the least apt to interfere with the further chemical procedure, and, in fact, treatment with alcohol represents a step in the process.

In the selection of the chemical methods for this series two points were kept in mind:

1. The necessity of correlating the chemical observations with the known facts of structure, to the interpretation of which they should add a greater precision. As an example of this, there were studied the sulphatides (lipoid sulphur) which are intimately associated in the nervous system with the sheaths of the medullated nerve fibers.

2. The collection of data, which, correlated with function, would give the physiologist a better knowledge of the nature of his material and thus enable him to do more than speculate as to the probable nature of the processes involved in the phenomena

he is, observing. As an example of this, there was studied the ratio between neutral sulphur and protein sulphur, a ratio which correlates closely with the decrease in metabolic activity associated with the growth of the nervous system from birth to maturity.

The general plan of the chemical technique has been first to block out the material into larger groups of substances and then carry the procedure of separation into greater detail. The necessity of working with data which represent something definite from the point of view of the chemist, has also been kept in mind.

The following outline illustrates the extent to which the chemical procedure has been carried up to the present.

*Outline illustrating the separation of constituents by the method employed,\* classified according to their state of aggregation.*

	ENCEPHALON DIVIDED BY STATE OF AGGREGATION INTO:			
	COLLOIDAL (FRACTION 1 AND 4)		NON-COLLOIDAL (FRACTION 2 AND 3)	
	Proteins (Fract. 4)	Lipoids (Fract. 1)	Organic Extractives (Fract. 2 and 3)	Inorganic Constituents (Fract. 2 and 3)
Proximate constituents	(Include supporting structures)	Phosphatides Cerebrosides Sulphatides { Cholesterol Undetermined		Sodium Potassium Calcium Magnesium Chlorides
Sulphur combinations	Protein S	Lipoid S	Neutral S	Inorganic S (sulphates)
Phosphorus combinations	Protein P	Lipoid P	Organic extractives P	Inorganic P (phosphates)

For an explanation of the chemical procedure followed for this separation the following outline has been inserted.

\* The method employed for this separation is described in an earlier paper by W. Koch and coworkers: *Journ. of the Amer. Chem. Soc.*, xxxi, pp. 1342-1361, 1909.

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*Moist Brain Tissue: Add alcohol and extract alternately with alcohol and ether.<sup>3</sup>*

EXTRACT (FRACTION 1 AND 2)		RESIDUE (FRACTION 3 AND 4)	
EVAPORATE TO DRYNESS, EMULSIFY WITH WATER, PPT. WITH $\text{CHCl}_3$ IN 0.5 PER CENT $\text{HCl}$ SOLUTION		DRY, WEIGH AND EXTRACT WITH HOT WATER	
Ppt. (Fract. 1):	Filtrate (Fract. 2):	Filtrate (Fract. 3):	Residue (Fract. 4):
Lipoids	Organic extractives Inorganic constituents	Organic extractives Inorganic constituents	Proteins

Organic extractives in Fraction 2 and 3 are equal to total organic extractives.

Inorganic constituents in Fraction 2 and 3 are equal to total inorganic constituents.

Fraction 1 and 2 are soluble in alcohol (85–95 per cent).

Fraction 3 is insoluble in alcohol; soluble in hot water.

Fraction 4 is insoluble in alcohol and hot water.

For a clearer understanding of the terms used in this series of papers, the following interpretation of the *chemical nature*, *anatomical distribution*, and *physiological significance* of the substances determined, with special reference to the nervous system based both on the studies already made and those presented in this paper, is given below.

### *Proteins.*

*Chemistry.* These represent complex combinations of amino-acids rendered insoluble in water by coagulation with hot alcohol. This fraction has been exhaustively extracted with hot alcohol and should retain only traces of lipoids and fats. The nucleoproteins and the neurokeratin are included in this fraction.

*Anatomical distribution.* In the part of the nervous system rich in cells (cortex) the proportion of the proteins is larger than in the white matter. Some of the nucleoproteins are supposed to be associated with the chromatin and Nissl substance of the nerve cell. The remainder of the nucleoproteins are represented by the nuclei of the glia cells scattered through-

<sup>3</sup> Although ether is used in the extraction following the first alcohol, it does not remove any considerable amount of material and need not be considered in the above scheme.

out the nervous system. Neurokeratin occurs in the medullated sheath of the nerve fiber. The other proteins occur in the axon of the nerve fiber as well as in the cell body and its dendrites.

*Physiological significance.* The proteins have usually been considered as the essentially living part of the protoplasm, but some of them, like neurokeratin, are undoubtedly inactive and represent supporting structures. The same may be said of the proteins which make up the fibers of the glia cells. It is therefore impossible to tell at the present time to just what extent and in what proportion the proteins are involved in the chemical activities of the nervous system. The significance of the neutral sulphur compounds, which represent simpler cleavage products of the larger protein aggregates, will be discussed later as having an important bearing on this point (see p. 431).

#### *Phosphatides.*

*Chemistry.* These represent complex combinations of fatty acids, phosphoric acid, glycerin, and nitrogen complexes of the nature of choline, and include among other things lecithin and kephalin. The chemistry of this group is very much in need of revision, as some of its members are not so simple as the older work of Hoppe-Seyler has led us to infer. The group does not include lecithin in combination with sulphur or cerebrin. The phosphatides as here given are calculated from the phosphorus of the lipid fraction on the assumption that they have an average molecular weight of 800. Correction must be made for the phosphorus of the sulphatides.<sup>4</sup>

*Anatomical distribution.* Comparison of cortex and corpus callosum<sup>5</sup> indicates that the phosphatides are not very differently distributed between cell body and nerve fiber. Analyses of the brain at a period when medullation has not begun, but when the cell processes are growing freely, indicate that the phosphatides are largely associated with the axon. If mitochondria consist largely of phosphatides, as has been suggested, the observations of Cowdry would give us a picture of their distribution in the cell body. The absence of mitochondria in the axon, which is known to contain phosphatides, would not argue against this, as there is some evidence that the phosphatides of the processes and the cell body are different in their behavior.

*Physiological significance.* The phosphatides, like the proteins, may be considered to be intimately associated with the vital processes of the living protoplasm. Their colloidal nature and relation to inorganic ions, as well

<sup>4</sup> *Calculations for phosphatides.* The total lipid phosphorus found times 25.77 gives the phosphatides, on the basis that 3.88 per cent of the phosphatides consist of phosphorus. Since 51.2 per cent of the sulphatides are phosphatides, that amount was deducted from the total phosphatides found. The difference was considered as free phosphatides.

<sup>5</sup> Koch, W.: *Amer. Journ. of Physiol.*, xi, pp. 326-328, 1904.

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as their instability towards heat,<sup>6</sup> lend support to this idea. They probably occur largely in the cytoplasm, cell body and its branches, where they may act as oxygen carriers, as has been suggested by the work of Koch and Mostrom.<sup>7</sup>

### *Cerebrosides.*

*Chemistry.* Complex combinations of fatty acids, galactose, and possibly other hexoses with a nitrogen complex of the nature of sphingosine. The cerebrosides are calculated from the lipid sugar on the assumption that they yield on hydrolysis 21.8 per cent of reducing sugar, the amount found by Thierfelder in his cerebrin. Correction must be made for the cerebrin content of the sulphatides.<sup>8</sup>

*Anatomical distribution.* Although the cerebrosides are occasionally met with in other tissues, they occur in largest amount in the medullated nerve fiber, and their quantity increases as medullation proceeds. The rather large amount found in the cortex<sup>9</sup> on chemical analysis indicates that they may predominate in the fibers of that region.

*Physiological significance.* As laid down in the medullated nerve fiber, the cerebrosides most probably serve only a mechanical function and are not available as sources of energy in spite of their carbohydrate and fatty acid content.

### *Sulphatides.*

*Chemistry.* These represent the combination of a phosphatide with a cerebroside by means of a sulphuric acid group in ester combination.<sup>10</sup> The sulphatides are estimated from the lipid sulphur on the basis of a sulphur content of 2 per cent, based on the analysis of a purified compound.<sup>11</sup>

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<sup>6</sup> Koch, W and Koch, M. L.: this *Journal*, xiv, pp. 281-282, 1913.

<sup>7</sup> Koch, W and Mostrom, H. T.: *Journ. of Pharm. and Exp. Ther.*, ii, No. 3, p. 265, 1910.

<sup>8</sup> *Calculations for cerebrosides.* The cerebrosides, from the lipid fraction, on hydrolysis for twenty-four hours with a weak solution of HCl (75 cc. of water containing 3 cc. concentrated HCl), yield 21.8 per cent by weight galactose. The calculations for cerebrosides were made on the assumption that galactose and glucose were equivalent in reducing power and the weight of galactose was thus determined from Munson and Walker's tables for glucose. (*Journ. Amer. Chem. Soc.*, xxviii, p. 663). The corrected weight of total galactose to cerebrosides was then made on the basis that 21.8 per cent of the latter is galactose. Finally since 42.9 per cent of the sulphatides consist of cerebrosides this amount was deducted from the total cerebrosides found. The difference was considered as cerebrosides.

<sup>9</sup> Koch, W. and Mann, S. A.: *Archives of Neurology and Psychiatry*, iv, p. 33, 1909.

<sup>10</sup> Koch, W.: *Zeitschr. f. physiol. Chem.*, lxx, p. 94, 1910.

<sup>11</sup> *Calculations for sulphatides.* These are considered to be of the general formula:



If it were desirable to recognize the chemical identity of the much abused protagon, the sulphatides might be considered as purified products. Protagon could be much more safely calculated from the lipid sulphur than from the lipid sugar as Noll<sup>12</sup> has attempted. The sulphur content of protagon preparations, when it has not been simply ignored, is variously reported as 0.5 and 1.0 per cent.

*Anatomical distribution.* The sulphatides, like the cerebrosides, increase parallel with the growth of the medullary sheath and may be considered as essential constituents of that structure. The fact that the sulphatides, as the result of more recent work, have been found to be pretty generally distributed in other tissues, indicates that they might occur in the cell body of the neurone, although a comparison of the analyses of cortex and corpus callosum does not make this very probable. The sulphatides have an important function in the maturing of the nerve fiber and give the Weigert staining reaction in a very characteristic manner.

*Physiological significance.* Their colloidal nature and the peculiar combination into which the sulphatides enter with potassium, suggests that they may have an important relation to the nerve impulse and to the phenomena of conductivity in general.

#### *Organic extractives and inorganic constituents.*

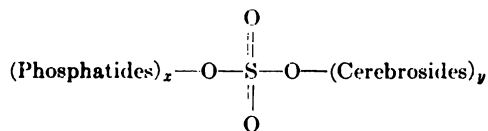
*Chemistry.* This group represents essentially the water-soluble, non-colloidal constituents of the nervous system. The older method of estimating the inorganic constituents by the ash has been abandoned as too inaccurate. The principal reason for reporting the above group is to give an idea of the ratio between the colloidal and non-colloidal constituents.

*Anatomical distribution.* The group occurs in large quantity in the cell body, although some is also present in the axon of the nerve fiber.

*Physiological significance.* This group is a rough index of the amount of metabolic activity going on in the tissue, as it represents at the same time the end products of chemical activity, as well as the culture media from which the more complex combinations are built up.

#### *Undetermined (cholesterol).*

This fraction is represented in the nervous system to a certain extent by cholesterol, which has not been directly estimated. Besides this, how-



containing 2.0 per cent sulphur, 42.9 per cent cerebrosides, and 51.2 per cent phosphatides. Then we have,

$$\frac{(\text{Lipoid sulphur} \times 50)}{\text{weight of dry substance}} = \text{per cent of sulphatides in dry substance.}$$

<sup>12</sup> Noll, A.: *Zeitschr. f. physiol. Chem.*, xxvii, p. 370, 1899.

ever, all the errors of analysis, as well as of such calculations as are based on assumed factors, enter into this fraction. After accounting for the cholesterol in the brain of the 50 and 100 mm. pig fetus,<sup>13</sup> in which this was estimated directly by Mendel, there remained undetermined 2 to 3 per cent of the total solids. Considering the number of groups estimated, this is not a very discouraging result. (In other tissues, which contain little cholesterol, the undetermined is recorded as neutral fat.)

*Anatomical distribution.* Cholesterol is principally of interest as a constituent of the medullary sheath to which it adds a sort of mechanical stability. But it is present in the cell bodies also, possibly contributing to the cell membranes. According to Lorrain Smith<sup>14</sup> it is one of the substances responsible for the color which the medullary sheath gives with Weigert's stain.

#### *Total sulphur and total phosphorus.*

It may not be out of place to state briefly the reasons for selecting these two elements for special determination in preference to others. As far as the phosphorus is concerned, the importance of the nucleins to all living cells and the phosphatides to the nervous system in particular, amply justify its selection. The reason for selecting sulphur in preference to the much more generally studied nitrogen, may, however need a word of explanation.

Nitrogen is studied for two reasons: because it is an important element in the building up of the proteins, and because it is easy of estimation.

Sulphur is just as characteristic of proteins, in fact more so, as it does not enter into the non-protein groups such as the nucleic acids. Among the lipoids, too, sulphur enters into only one group, the sulphatides, while nitrogen occurs in all except cholesterol.

In other words, to estimate sulphur in the protein fraction is to estimate an element essentially characteristic of the more truly protein part. To estimate it in the lipid fraction, enables one to distinguish one particular, and, as growth curves show, a very interesting group of lipoids. Besides, as has already been pointed out in a previous paper<sup>15</sup> sulphur occurs in the tissues in several states of oxidation and thus gives us some indication of the intensity of reactions of oxidation which are so important to growing tissues, and about which we know so little. It seems wise therefore to estimate the sulphur, and in case there are any special reasons to study nitrogen, to study it rather in the form of one of its definite groups of compounds such as the purine bases or the amino-acids.

<sup>13</sup> Koch, Mathilde L.: this *Journal*, xiv, pp. 267-279, 1913.

<sup>14</sup> Smith, Lorrain: *Journ. of Path. and Bact.*, xv, pp. 179-181, 1911.

<sup>15</sup> Koch, W. and Upson, F. W.: *Proc. Soc. for Exp. Biol. and Med.*, vii, pp. 5-6, 1909.

*Distribution of sulphur.*

**Chemistry.** **PROTEIN S.** This group represents sulphur in various amino-acid combinations such as cystine or cysteine. The proteins in which this sulphur fraction is found have been coagulated and rendered insoluble in water by treatment with hot alcohol.

**LIPOID S.** Ethereal sulphuric acid combinations are discussed under sulphatides.

**NEUTRAL S.** This group of compounds represents the total non-colloidal, water-soluble combinations of sulphur, minus the inorganic sulphates. As far as studied, they resemble in all their reactions a similar group found in the urine and called by Bondzynski proteinic acids. They represent, probably, larger cleavage products of the protein molecule or complex non-coagulable, water-soluble polypeptides somewhat altered by processes of oxidation. The sulphur of this fraction is represented essentially by compounds included among the organic extractives, and the sulphur is most often in an oxidized form like taurine or ethereal sulphate.

**INORGANIC S** (inorganic sulphates). Derivatives of sulphur directly precipitated by barium chloride in hydrochloric acid solution.

*Anatomical distribution and physiological significance.* The **LIPOID S**, as has already been mentioned under the sulphatides, represents an essential constituent of the medullary sheath. The proportion in which it occurs in the sheath can be considered as a measure of the maturity of the sheathing substance.

**THE PROTEIN S AND NEUTRAL S** will be considered together as they bear an important relation to one another and as the combinations in which they occur are essential constituents of all living cells. As has already been stated, the study of these two groups of sulphur compounds gives us a means of investigating the protein metabolism of the central nervous system during its growth period.

TABLE I.

*A comparison of neutral sulphur with protein sulphur in the brain of the albino rat at different ages (figures in per cent of total sulphur).*

	PROTEIN SULPHUR	NEUTRAL SULPHUR
1 day.....	30.5	48.2
10 days.....	44.2	45.4
20 days.....	56.4	28.6
40 days.....	63.7	18.2
120 days.....	61.8	18.7
210 days.....	63.8	14.5

During the early stages when growth is proceeding rapidly and chemical activities may be considered to be at their height, the proportion of

non-colloidal, relatively smaller, neutral sulphur molecules is at a maximum. This is what we should expect when we consider living matter not as a collection of highly organized molecules, but rather as a heterogeneous substratum in which relatively smaller molecules or their dissociated products are engaged in chemical transformations. As the tissues grow and become more highly differentiated and mature, more and more protein is laid down as structural material, and the proportion is shifted in the direction of the protein sulphur. A comparison of the cortex of the human at two years and at maturity illustrates this point.<sup>16</sup>

2 years' cortex.....	Protein S, 63; Neutral S, 22.
19 years' cortex.....	Protein S, 73; Neutral S, 12.

The change suggests, therefore, a decrease in chemically active material associated with the increasing complexity of the tissue. Such data as we have at hand indicate that we have in the protein sulphur and neutral sulphur ratio a valuable means of measuring the relative growth intensity of the nervous system at different periods during its development after the state of cell division has practically ceased.

There might be another way of measuring this intensity of chemical activity, namely, by means of the inorganic sulphates, which represent the end products and the final state of oxidation of the compounds involved in these reactions, but they are eliminated rather easily from the cell, and it is therefore difficult to attach any significance to their variations.

#### *Distribution of phosphorus.*

**Chemistry. PROTEIN P.** This group represents phosphorus largely in combination as nucleic acid. In the nervous system this nucleic acid is combined with such a very large amount of protein<sup>17</sup> that the per cent of phosphorus in the resulting nucleoprotein drops to 0.57 per cent as compared with 3 to 4 per cent in such a tissue as the pancreas.

**LIPID P.** Already discussed under phosphatides.

**WATER-SOLUBLE P.** This group includes non-colloidal, water-soluble organic combinations of phosphoric acid and inorganic phosphates. On account of the relative ease with which the organic extractive combinations of this form of phosphoric acid break down, it is difficult to estimate the proportion which is in organic combination. The results which are so far recorded represent, therefore, rather the possible maximum, than a very close approach to the actual value. (See articles of Grindley,<sup>18</sup> Trowbridge,<sup>19</sup> and Forbes.<sup>20</sup>)

<sup>16</sup> Koch, W. and Mann, S. A.: *Journ. of Physiol.*, xxxvi, p. 2, 1907.

<sup>17</sup> Also accounts for poor staining reaction of neurone nucleus.

<sup>18</sup> Grindley: *Journ. of Amer. Chem. Soc.*, xxviii, pp. 25-63, 1906.

<sup>19</sup> Trowbridge, P. F. and Francis, C. K.: *This Journal*, vii, pp. 481-501, 1910.

<sup>20</sup> Forbes, E. B.: *Ohio Agric. Exp. Sta. Bulletin* 215, 1910, pp. 459-489.

**Anatomical distribution and physiological significance.** The *protein phosphorus* is largely associated with the nucleic acid of the nucleus. In the nervous system it is also supposed to be associated with the Nissl substance, but this is still a doubtful matter. The accuracy with which we can estimate nuclear material in the anatomical sense from such a figure as the protein phosphorus is difficult to determine, as there are three complicating factors.

1. The possibility that the nucleus, as an anatomical unit, contains other compounds besides nucleoproteins.

2. The fact that nucleic acid itself may be associated with very widely varying quantities of protein.

3. The possibility that substances yielding a protein phosphorus fraction may occur in the cytoplasm.

Observations by Miescher<sup>21</sup> on the sperm, however, very strongly suggest that protein phosphorus is largely associated with the nucleus, while lipid phosphorus is largely associated with the cytoplasm.

As regards the *water-soluble phosphorus*, the principal point of interest, just as in the case of the neutral sulphur, is its ratio to the protein or colloidal forms of phosphorus. Thus in a study on a lower plant form (*Aspergillus niger*) Koch and Reed<sup>22</sup> could demonstrate that under extreme conditions, such as can only be realized with plant material, it is possible to carry the growth processes to such a point that all the non-colloidal, water-soluble phosphorus is converted into colloidal combinations. At such a point the growth of the plant comes to a stop.

The function of the *inorganic phosphates* in maintaining the neutrality of protoplasm as suggested by Henderson<sup>23</sup> is also a point of interest, although of less importance to the nervous system than to muscle tissue.

#### *Inorganic constituents.*

**Chemistry.** The inorganic constituents found in the nervous system are the cations Na, K, Ca, Mg, Fe, and the anions Cl, SO<sub>4</sub>, PO<sub>4</sub>.

In the method devised for this study the usual method of estimating these constituents by the ash was abandoned on account of the fact that by the process of ashing, the relation of cations to anions is profoundly altered. As a result of this precaution, the interesting fact has been clearly demonstrated that a large proportion of the cations, especially sodium and potassium, occur combined with complex anions, sometimes colloidal in nature.

The work of Pike<sup>24</sup> has brought to light the interesting point that in the nervous system the sodium and potassium, more especially the latter,

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<sup>21</sup> Miescher, F.: *Hoppe-Seyler's Med.-chem. Unters.*, p. 452.

<sup>22</sup> Koch, W. and Reed, H. S.: *this Journal*, iii, p. 49, 1907.

<sup>23</sup> Henderson, L. J.: *this Journal*, vii, pp. 29-35, 1910.

<sup>24</sup> Koch, W. and Pike, F. H.: *Journ. of Pharm. and Exp. Ther.*, ii, pp. 245-248, 1910.

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are combined with such lipoids as the sulphatides and kephalines (a subgroup of phosphatides), while the Ca and Mg have more tendency to remain combined with the proteins.

*Anatomical distribution and physiological significance.* Very little is known of the anatomical distribution of the salts except as shown by the work of Macallum<sup>25</sup> which demonstrates that chlorides and potassium are associated with the nerve fiber. According to Alcock,<sup>26</sup> potassium is supposed to play an important rôle in the propagation of the nerve impulse.

With this introductory statement of the anatomical distribution and physiological significance of the substances quantitatively determined, we may now present the results of a study of their variation during the growth of the brain.

The brain of the albino rat was selected for this study, for the reasons already presented in the first paper of this series.<sup>27</sup>

From a comparison of the brain of the albino rat at birth and the brain of the fetal pig, it was found that the brain of the new born rat is as young nervous material as can conveniently be analyzed at present. It forms therefore a suitable starting point for this study of chemical differentiation during growth. The analyses reported in this paper are those of the brains of rats aged respectively, 1, 10, 20, 40, 120, and 210 days. The results show that it was possible to follow closely the various structural changes which occur during the differentiation of the growing nervous system

The material was furnished by the Wistar Institute of Anatomy; the brains being collected and analyzed in the manner already detailed.<sup>28</sup> Koch's quantitative methods were used.<sup>29</sup>

### RESULTS OF ANALYSES.

The results of analyses are embodied in Table II. Duplicate analyses have been carried on throughout, and are summarized in Table III. This table gives the averages of the analyses, except

<sup>25</sup> Macallum, A. B.: *Journ. of Physiol.*, xxxii, pp. 95-128, 1905; Macallum, A. B. and Menten, M. L.: *Report 75th Meeting British Assoc. Adv. Sci.*, p. 555, 1906.

<sup>26</sup> Alcock, N. H.: *Journ. of Physiol.*, xxxix, pp. 402-410, 1911.

<sup>27</sup> Koch, Mathilde L.: *this Journal*, xiv, pp. 267-279, 1913.

<sup>28</sup> Koch, Mathilde L.: *loc. cit.*

<sup>29</sup> Koch, W.: *Journ. of Amer. Chem. Soc.*, xxxi, pp. 1335-1364, 1909.

in two instances where the value from one analysis only is preferred. Table IV gives the absolute weights of these constituents, as found in one brain; while Table V gives the ratio of increase of the different constituents, taking the amount of each constituent in the brain of the rat at birth to be unity and determining the number of times each constituent had increased at successive ages from birth to maturity. For comparison there is given in Table II one analysis of the spinal cord at 120 days.

#### DISCUSSION OF RESULTS.

The growth of the nervous system from the first laying down of the neural canal to maturity may be divided into four periods. The first period, during which cell division is the most characteristic feature, lasts to about birth. A short time before birth cell division begins to decrease. The chemical changes during this first period were not studied directly in the albino rat for the reasons stated in the first paper<sup>30</sup> but the composition of the nervous system in this primitive, undifferentiated state may be seen in the analysis of the fetal pig brain reported in the first paper of the series. At this time phosphatides are present, sulphatides are relatively less important and cerebrosides are entirely lacking; proteins, phosphatides, extractives, salts and water are the predominant constituents of the tissue.

The second period (see Table VI) lasts from birth for about ten days, when the third period begins. The second period is characterized structurally by the development of fibers from the cells and the increase in their size. Donaldson has estimated that the number of nerve cells does not increase more than 3 to 6 per cent during this period, but the cells do add to the number and size of their branching processes. This period, as may be seen from Table VI, is one of intense growth of all the solid constituents. The proteins continue throughout this period to be formed at a very rapid rate, 4-5 mgms. being laid down per day. Cerebrosides are either absent entirely, or present in very small quantities.

In the third period, that of most rapid growth, from the tenth to the twentieth day, medullation begins. There is a wonderful

<sup>30</sup> Koch, M. L.: this *Journal*, xiv, p. 279, 1913.





TABLE II. Continued.

*Distribution of sulphur in per cent of total S.*

Protein S.....	31.1	30.0	48.6	44.2	57.5	55.3	65.1	62.4	61.2	62.4	63.8	53.7
Lipoid S.....	3.2	2.8	2.2	6.1	6.7	7.5	9.2	10.1	12.8	12.5	15.6	30.9
Neutral S.....	49.1	47.3	45.1	45.4	29.7	27.5	17.0	19.3	19.2	18.3	14.5	10.3
Inorganic S.....	16.6	19.9	4.1	4.3	6.1	9.7	8.7	8.2	6.8	6.8	6.1	5.1

*Distribution of phosphorus in per cent of total P.*

Protein P.....	13.3		13.0	13.9	6.0	5.8	9.9	7.5	7.4	7.3	6.8	5.6
Lipoid P.....	33.2	33.0	33.8	36.1	52.2	53.5	56.1	58.5	65.8	62.3	67.6	77.4
Water Sol. P.....	53.5	53.6	53.2	50.0	41.8	40.7	34.0	34.0	26.8	30.4	25.6	17.0

\* Cerebrosidee not determined in brains at birth and 10 days. Probably none present at this age.

† By difference.

‡ Indicates doubtful result.

§ Taken from W. 8.

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TABLE III.

*The relative proportions of the constituents of the brain of the albino rat at different ages (averages from Table II).*

	AGE IN DAYS					
	1	10	20	40	120	210
Moist weight of one brain in grams..	0.25*	0.86†	1.28*	1.38*	1.60*	1.67†
Solids in per cent.....	10.42	12.5	17.5	20.34	21.65	21.9
Dry weight of one brain in grams....	0.026	0.107	0.224	0.281	0.347	0.365
Number of brains in each sample....	100	40	54	35	30	31
Laboratory Number.....	W. 16, 24	W. 40	W. 17, 25	W. 28, 29	W. 7, 8	W. 13

## *Constituents in per cent of total solids.*

Proteins.....	58.25*	56.5†	53.3*	48.4*	47.6*	48.5†
Phosphatides.....	15.2	12.3	21.4	21.8	21.6	22.0
Cerebrosides.....			3.0	5.9	8.4†	8.4†
Sulphatides.....	1.45	2.6	2.5	2.55	3.55	4.5
Organic extractives.....	17.9	15.1	14.55	14.85	9.75	9.8†
Inorganic constituents.....						
Cholesterol (undetermined)§.....	7.2	13.5	5.25	6.5	9.1	6.8
Total sulphur.....	1.00	0.83	0.70	0.55	0.56	0.58
Total phosphorus.....	1.87	1.48	1.66	1.52	1.42	1.39

## *Distribution of sulphur in per cent of total S.*

Protein S.....	30.5	44.2	56.4	63.75	61.8	63.8
Lipoid S.....	3.0	6.1	7.1	9.65	12.7	15.6
Neutral S.....	48.2	45.4	28.6	18.15	18.7	14.5
Inorganic S.....	18.3	4.3	7.9	8.45	6.8	6.1

## *Distribution of phosphorus in per cent of total P.*

Protein P.....	13.3	13.45*	5.9	8.7	7.3	6.8
Lipoid P.....	33.2	34.95	52.85	57.3	64.1	67.6
Water sol. P.....	53.5	51.6	41.25	34.0	28.6	25.6

\* Record from average duplicate analyses.

† Record from one analysis only.

‡ Taken from analysis, W. 8.

§ Obtained by difference.

TABLE IV.

*Absolute weights, in milligrams, of the constituents of a single brain of the albino rat at different ages (prepared from Table III).*

	AGE IN DAYS					
	1	10	20	40	120	210
Moist weight of one brain in grams	0.25	0.86	1.28	1.38	1.60	1.67
Solids in per cent	10.42	12.5	17.5	20.34	21.65	21.9
Dry weight of one brain in grams	0.026	0.107	0.224	0.281	0.347	0.365
Laboratory Number	W. 16, 24	W. 40	W. 17, 25	W. 28, 29	W. 7, 8	W. 13

*Absolute weights in milligrams.*

Proteins (1)†	15.14*	60.45†	119.4*	136.0*	165.2*	177.0†
Phosphatides (2)	3.95	13.16	47.9	61.3	74.95	80.3
Cerebrosides (3)			6.7	16.6	29.15	30.66
Sulphatides (4)	0.38	2.78	5.6	7.2	12.3	16.4
Organic extrac-	4.65	16.16	32.6	41.7	33.8	35.8
tives						
Inorganic constituents						
Cholesterol undetermined (5)	1.87	(14.45)	11.7	18.2	31.6	24.8
Total sulphur	0.26	0.90	1.57	1.54	1.94	2.12
Total phosphorus	0.48	1.6	3.72	4.30	4.93	5.07

*In absolute weight in milligrams of sulphur.*

Protein S (1S)§	0.079	0.398	0.885	0.982	1.199	1.352
Lipoid S (4)	0.008	0.054	0.111	0.149	0.246	0.330
Neutral S (6)	0.125	0.409	0.449	0.279	0.363	0.307
Inorganic S (7)	0.047	0.039	0.122	0.130	0.132	0.129

*In absolute weight in milligrams of phosphorus.*

Protein P (1P)	0.064	0.215*	0.230	0.374	0.360	0.345
Lipoid P (2)	0.161	0.558	1.964	2.464	3.160	3.427
Water sol. P (8)	0.260	0.826	1.532	1.462	1.410	1.298

\* Record from average duplicate analyses.

† Record from one analysis.

‡ Figures in parentheses in this section refer to Chart III.

§ Figures in parentheses in this and the following sections refer to Chart IV.

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TABLE V.

*The ratio of the increase of the constituents of the brain of the albino rat at different ages, taking the amount of each constituent found in the brain at birth as unity (prepared from Table IV).*

	1	AGE IN DAYS				
		10	20	40	120	210
Total Solids.....	1	4.0	8.6	10.8	13.3	14.0
Proteins.....	1	4.0	7.9	9.0	11.0	11.7
Phosphatides.....	1	3.3	12.0	15.5	19.0	20.3
Cerebrosides.....						
Sulphatides.....	1	7.4	14.8	19.0	32.6	43.5
Organic extractives.....	1	3.5	7.0	8.9	7.2	7.7
Inorganic constituents.....						
Cholesterol (undetermined).....	1	7.7	6.2	9.0	16.9	13.2
Total sulphur.....	1	3.4	6.0	5.9	7.4	8.0
Total phosphorus.....	1	3.2	7.6	8.8	10.1	10.4
Protein S.....	1	5.0	11.1	12.3	15.0	17.0
Lipoid S.....	1	6.9	14.3	19.1	31.5	42.3
Neutral S.....	1	3.3	3.6	2.2	2.9	2.5
Inorganic S.....	1	(1.3)	2.6	2.7	2.8	2.7
Protein P.....	1	4.1	4.3	7.2	7.0	6.7
Lipoid P.....	1	14.3	15.3	19.2	24.7	26.8
Water sol. P.....	1	3.9	7.4	6.0	6.8	6.2

TABLE VI.

*Rate of growth (milligrams formed per day) of different constituents in a single brain of the albino rat at different age periods (prepared from Table IV).*

	AGE PERIODS				
	2	3	4		
Between.....	1-10 days	10-20 days	20-40 days	40-120 days	120-210 days
Proteins.....	4.53	5.9	0.84	0.36	0.13
Phosphatides.....	0.92	3.5	0.67	0.17	0.06
Cerebrosides.....			0.49	0.15	0.006
Sulphatides.....	0.24	0.29	0.08	0.06	0.045
Organic extractives.....	1.51	1.64	0.46	0.00	0.000
Inorganic constituents.....					
Cholesterol (undetermined).....	(0.49)	(0.49)	0.32	0.17	0.000

outburst of activity in forming phosphatides which reach a maximum rate of formation of 3.5 mgms. per day. This change is no doubt correlated with the great growth of the fibers and the beginning of medullation. The organic extractives and inorganic constituents continue to be formed at the same rate since the cell bodies are increasing in size; probably not more than from 10 to 20 per cent having reached anything approaching adult size up to this time.

The sulphatides, although present in less quantity than the phosphatides, reach also their maximum rate of formation. The whole chemical picture is that of a rapid growth of protoplasm, with a change in its character owing to the increase of phosphatides. During this period the neurones increase rapidly in size.

Attention is particularly directed to the temporary great increase in neutral sulphur during these two periods of intense growth (Table IV). The significance of this has already been discussed on p. 431 *et seq.*

The fourth growth period is the period of continued medullation. This period is characterized chemically by a great reduction in the rate of formation of all substances except the cerebroside. These latter between 20-40 days come into view, almost equalling the phosphatides and being more than half the amount of the proteins formed at the same time. The cerebroside contributes a large share toward medullation. The rate of formation of the various constituents per day falls in the 20-40 day period, as compared with the 10-20 day period, in the case of the proteins to one-seventh; the phosphatides to one-fifth; the sulphatides to one-third; and the organic and inorganic extractives to one-third. The formation of the proteins decreases the most; the cerebroside, the least. If the rate of formation in the 40-120 day period is compared to that of the 10-20 day period it is seen that the protein formation has decreased to one-sixteenth; the phosphatides to one-twentieth; the sulphatides to one-fourth but are still increasing. On the other hand, the organic extractives and inorganic constituents have not increased at all, indicating that metabolism is much reduced in its rate and the growth of the protoplasm is much slower. During this fourth period then, the sulphatides continue to be formed at a more rapid rate, relative to their total amount, than any other constituents; and in the 120-210

day period, the total sulphatides formed surpass the cerebroside, nearly equal the phosphatides, and are more than one-third the proteins. The constant production of sulphatides is, therefore, a marked feature of late medullation, just as that of the phosphatides is of the early medullation. The sulphatides diminish in their rate of formation far less than any other constituents.

Finally we have the period from 210 days on: the period of stationary or adult life. We have no definite chemical data as to any changes occurring during this period, but from such data at hand, as the periods just studied, we can assume that the growth processes during adult life are practically stationary except perhaps a very gradual increase in the per cent of solids.

The enlargement of the brain may, therefore, in great part be accounted for chemically by the formation of the medullary sheath. Donaldson<sup>31</sup> has found that some 88 per cent of the volume of the adult brain is composed of the axons and their sheaths, while the cell bodies with their dendrites and the supporting tissues together only make up the remaining 12 per cent. The axones, therefore, medullated or non-medullated, are mainly responsible for the increase of the size of the brain and for the changes which it undergoes during post natal growth. To bring more vividly before the eye the relative rate of growth of the various constituents, we have prepared Charts 1 and 2 from Tables III and IV. These charts are self explanatory. We have also prepared Charts 3 and 4, an explanation of which is given below.

Chart 3 shows the relation of lipoid<sup>32</sup> to protein. In this the weights of the several constituents are represented for one brain at each age. This chart shows that while both the proteins and the lipoids are increasing in absolute weight, the proportion of lipoid to protein is becoming greater and greater as the tissue grows older. This indicates that the rate of increase for the lipoids is greater than for the proteins (also brought out in Table VI). At 120 and at 210 days we find that the lipoids and pro-

<sup>31</sup> Donaldson, H. H.: *Journ. of Nervous and Mental Disease*, xxxviii, p. 260, 1911.

<sup>32</sup> Lipoids here include the phosphatides, cerebroside, and sulphatides; cholesterol, which is classed as lipoid, is here recorded in the "undetermined."

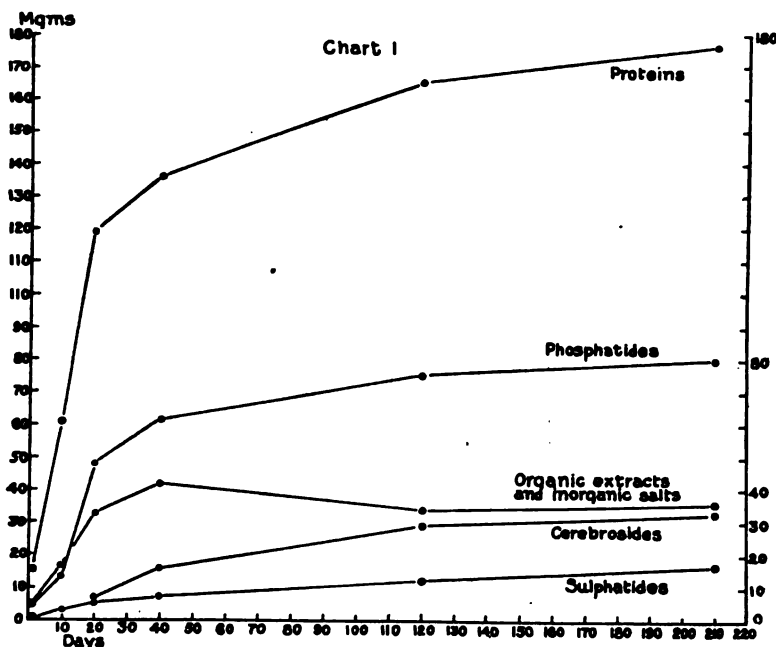


CHART 1. Shows the absolute weight in milligrams, of the constituents of a single brain of the albino rat at different ages. (Age in days along the abscissa; mgms. on the ordinate.)

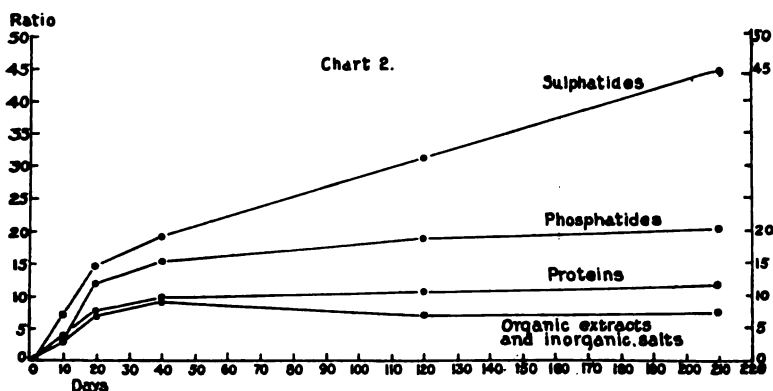


CHART 2. Shows the rate of increase of the different constituents of the brain of the albino rat at different ages, taking the amount of each constituent in the brain at birth as unity. (The ordinate shows how many times the weight of each constituent has increased over its amount at birth at the age plotted on the abscissa.)

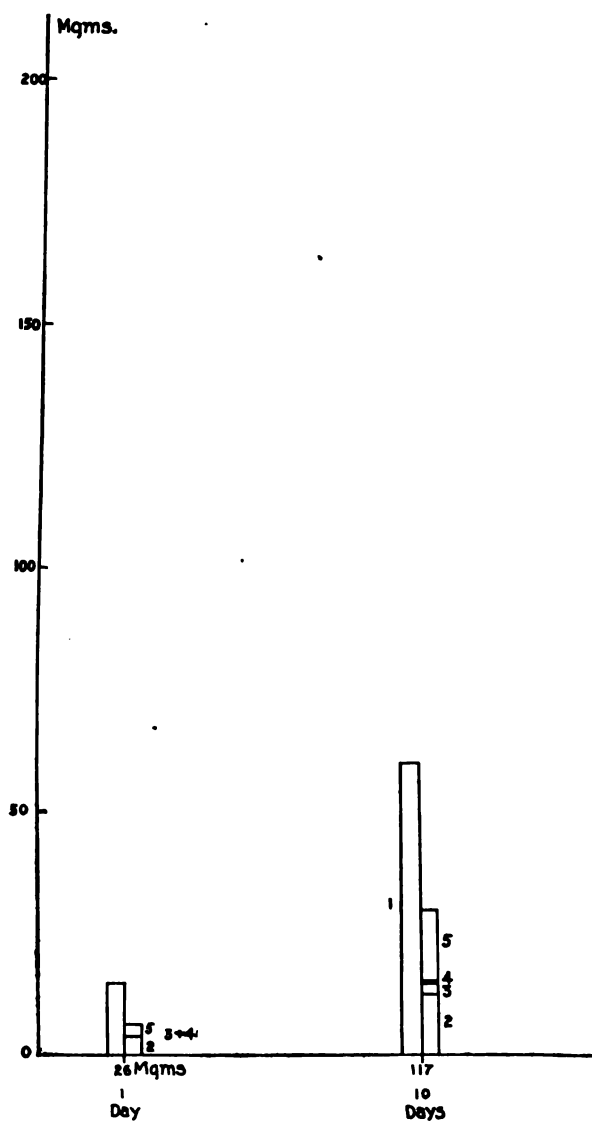
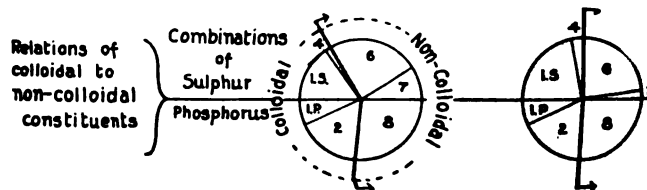
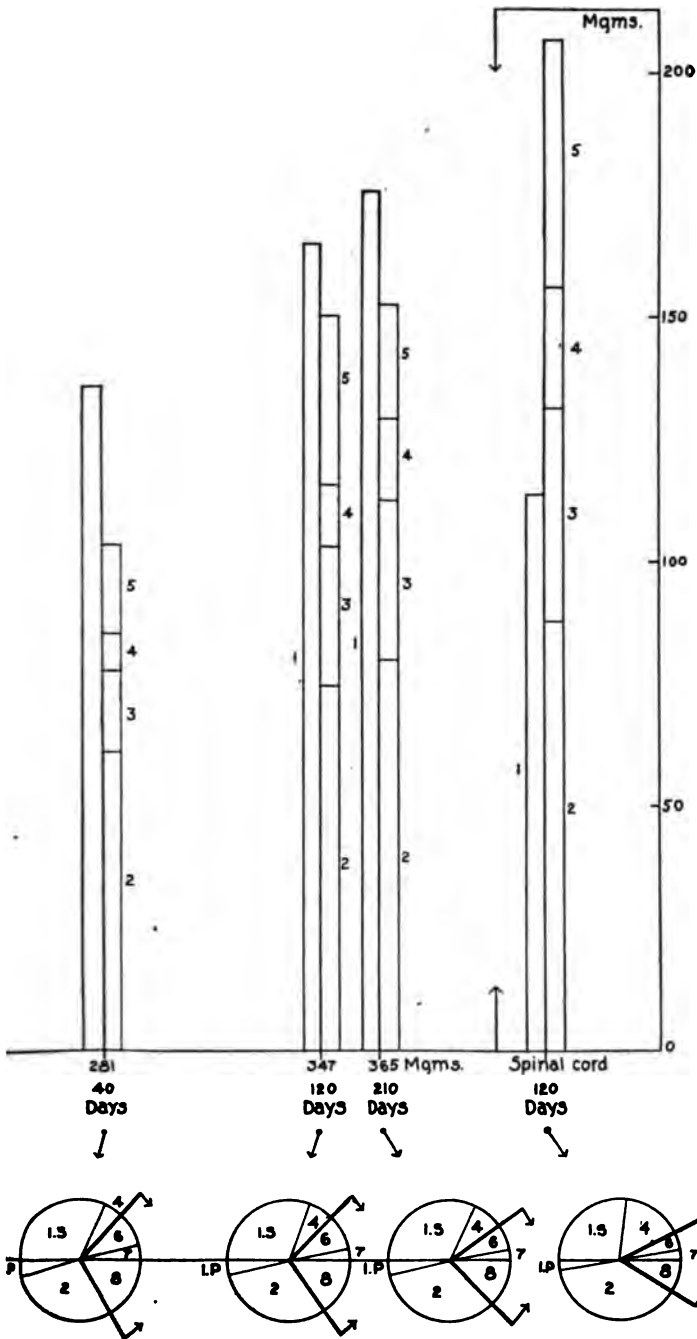


Chart 4







● **CHART 3.** Shows, in absolute weight, the amounts of proteins and of lipoids in the brain of the albino rat at different ages. Based on Table IV. 1, Proteins; 2, Phosphatides; 3, Cerebrosides; 4, Sulphatides; 5, Undetermined lipoids (Cholesterol). The organic extractives and inorganic constituents are not included. For comparison the weights of the several constituents in the spinal cord of the albino rat at 120 days are also shown; the weight of the dry substance of the cord being taken as equal to that of the 120-day brain.

**CHART 4.** Shows, in the albino rat at different ages, the proportional values in segments of a circle for the sulphur combinations (above the equator) and for the phosphorus combinations (below the equator); with a further grouping into colloids and non-colloids. Based on Table IV. Again for comparison the proportional values for the sulphur and the phosphorus combinations in the spinal cord of the albino rat at 120 days are also given.

*Sulphur combinations:* 1, Proteins (protein sulphur); 4, Sulphatides (lipoid sulphur); 6, Neutral sulphur (proteic acids); 7, Inorganic sulphates.

*Phosphorus combinations:* 1, Proteins (protein phosphorus); 2, Phosphatides (lipoid phosphorus); 8, Organic and inorganic phosphates (water soluble phosphorus).

The colloids are represented by 1 and 4, of the sulphur combinations and 1 and 2 of the phosphorus combinations.

The non-colloids are represented by 6 and 7 of the sulphur combinations and by 8 of the phosphorus combinations.

teins are present in the brain in nearly equal proportions. For the sake of comparison, the corresponding values for the spinal cord at 120 days have been introduced into this chart.

To make easier the comparison between the relations of proteins and lipoids in the brain and in the spinal cord, it is assumed for the purposes of the chart that the dry weight of the cord is the same as that of the brain at 120 days. Since the cord contains a larger proportion of white matter than does the brain, we find that the lipoids in this case predominate over the proteins. This indicates that the chemical differentiation during the growth of the nervous system, as recorded in this paper, is largely concerned with the development of the medullated nerve fiber.

Chart 4 shows very strikingly the great decrease with advancing age in the non-colloidal contrasted with the corresponding increase in the colloidal sulphur and phosphorus compounds. Particular attention is called to the neutral sulphur which in the young, rapidly metabolizing tissue constitutes the greater proportion of the total sulphur, whereas it becomes extremely small at 210 days when growth metabolism is at an end. Evidently this fraction may, with reserve, be considered an index of growth metabolism. With advancing age the colloidal, less active, substances gradually crowd out the non-colloidal. This is in striking accord with the interesting suggestions of Child<sup>33</sup> that senescence is due to the accumulation of these colloidal solids, which interpose resistance to metabolism.

Finally we find the growth process characterized by a steady diminution in the proportions of water and an increase in the proportion of solids. This change is due not alone to medullation in the strict sense, since as Donaldson<sup>34</sup> has pointed out the decrease begins before medullation, between birth and ten days in the rat. He attributes it to a rapid growth of the axone at this time. Water and the proportion of neutral sulphur are therefore criteria of the youthfulness of tissue, while the increase of lipid sulphur (sulphatides) is a criterion of medullation.

<sup>33</sup> Child, C. M.: *Archiv. f. Entwicklungs-mechanik d. Organismen*, xxxi, p. 571, 1911.

<sup>34</sup> Donaldson, H. H.: *Journ. of Neurology and Psychology*, xx, p. 138, 1910.

## SUMMARY.

The principal results of this study may be summarized as follows:

Well-marked and characteristic chemical changes occur in the rat-brain during its growth and these changes are obviously correlated with its anatomical differentiation.

The principal chemical changes noted are:

1. A general decrease in the per cent of water which is not due entirely to medullation since the decrease begins before medullation (Donaldson '10).

2. A diminution in the relative per cent of protein in the total solids due to the formation of a large amount of lipoid matter.

3. The lipoids which appear coincident with medullation and of which the development is *pari passu* with medullation are the cerebroside and sulphatides. These, therefore, are chiefly found in the medullary sheaths.

4. There is a great outburst of phosphatide formation at the very beginning of medullation, but the phosphatides are present also in large amounts before medullation. The phosphatides are present, therefore, in the cells as well as the sheaths.

5. The extractives are present in largest amounts during fetal and early life when growth and metabolism are at a maximum. Particularly the water-soluble, organic sulphur compounds (neutral sulphur) diminish relatively with age, while the colloidal sulphur increases. The relations of the neutral sulphur may be interpreted, therefore, as indicating the intensity of metabolic activity.

6. The great increase of colloidal matter with age clearly indicates that this, in the form of supporting structures, constitutes a relatively inactive material which presumably serves to localize chemical processes. The accumulation of this material is probably one factor producing the general slowing of metabolism characteristic of senescence. This would thus become one cause of senescence as Child has suggested.

## ON THE PRESENCE OF ADENASE IN THE HUMAN BODY.

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The individuality of the two deaminizing enzymes concerned in purine metabolism, guanase and adenase, can be considered established. The former contention of Schittenhelm that one deaminizing ferment converts guanine into xanthine and adenine into hypoxanthine, can no longer hold, in the light of accumulated evidence of the ability of some organs to convert one of the purines without affecting the other. Of these two enzymes guanase is much more widely distributed in animal tissues. It is still a disputed question whether or not adenase occurs in the human body at all.

The method which has usually been applied in the study of purine enzymes is the subjection of purines *in vitro* to the action of extracts of animal tissues. How well this method represents the actual processes as they take place within the living organism, cannot be said. Such experiments should be supplemented where possible by a study of the fate of purines introduced within the living body, and experiments of this kind are recorded in the literature.

The latest investigations of the action upon adenine of extracts of human tissues may be summarized as follows: In an article published in 1907 Schittenhelm and Schmid<sup>1</sup> reported that added adenine was destroyed after seventeen days' incubation with extracts of human liver, intestine, and thymus, no purines at all being recovered, and that adenine was converted into hypoxanthine by extract of muscle and kidney, and that it was not attacked by extract of lung. In a later investigation Schittenhelm<sup>2</sup> revised his findings, obtaining evidence that adenine is converted into

<sup>1</sup> *Zeitschr. f. exp. Path. u. Ther.*, iv, pp. 424-431, 1907.

<sup>2</sup> *Zeitschr. f. physiol. Chem.*, lxiii, pp. 248-268, 1909.

hypoxanthine by extract of human lung, and to a less extent by extracts of kidney and intestine, inconclusive results being reached with liver and muscle. He asserts, in opposition to the contention of Jones and his co-workers, who in a long series of investigations have reached the conclusion that adenase is not present in the human body, that human tissues have in varying degree the ability of converting adenine into hypoxanthine. Winternitz and Jones<sup>3</sup> found that adenine could be recovered quantitatively after treatment with extracts of human liver and spleen, and Miller and Jones<sup>4</sup> found that adenase was not present in the spleen, liver, pancreas, kidney, or lung and claim that the enzyme is not contained in human tissue at all. Wells and Corper,<sup>5</sup> on the other hand, found that extracts of fetuses of the five and six month stages converted adenine, but that the extract of a three months' fetus did not, and furthermore that adenine could not be recovered, while hypoxanthine was obtained in quantity, in the autolysis of a fetus of the sixth month. That this last, however, can be taken in another light than as indicating the presence of adenase, will be seen from the following:

It has been repeatedly proved that hypoxanthine is not necessarily a product of the action of adenase upon adenine, and may occur free in tissues containing no adenase. This Jones calls "preformed hypoxanthine." Leonard and Jones<sup>6</sup> found that pig and rabbit muscle, in which adenase is not present, nevertheless contain large amounts of hypoxanthine, and further that hypoxanthine is always one of the products of autolysis of human spleen, although this tissue contains no adenase; that is, its extract will not convert adenine into hypoxanthine. After similar experience with other tissues Leonard and Jones and Vögtlin and Jones<sup>7</sup> were led to believe that "preformed hypoxanthine" was present in all tissues. Straughn and Jones<sup>8</sup> demonstrated its presence in yeast, which does not contain adenase. Wells and Long<sup>9</sup> found that in the prolonged autolysis of human tumors which did not contain adenase, no adenine could be recovered, while great amounts of hypoxanthine were formed. Quite recently Amberg and Jones<sup>10</sup> have shown that hypoxanthine may be formed in the splitting of nucleic acid by enzymes, without passing through the adenine stage, adenosine and inosine being the intermediate products.

Experiments on the fate of adenine fed to human beings are not so numerous. Krüger and Schmid<sup>11</sup> fed adenine to a man and found an

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<sup>3</sup> *Zeitschr. f. physiol. Chem.*, lx, pp. 180-190, 1909.

<sup>4</sup> *Ibid.*, lxi, pp. 395-404, 1909.

<sup>5</sup> *This Journal*, vi, pp. 469-482, 1909.

<sup>6</sup> *Ibid.*, vi, pp. 453-460, 1909.

<sup>7</sup> *Zeitschr. f. physiol., Chem.*, lxvi, pp. 250-256, 1910.

<sup>8</sup> *This Journal*, vi, pp. 245-255, 1909.

<sup>9</sup> *Zeitschr. f. Krebsforschung*, xii, pp. 598-611, 1913.

<sup>10</sup> *Zeitschr. f. physiol. Chem.*, lxxiii, pp. 407-415, 1911.

<sup>11</sup> *Ibid.*, xxxiv, pp. 549-565, 1902.

increase in the elimination of uric acid corresponding to 41 per cent of the nitrogen of the adenine fed. They also noticed an increase of 3 per cent in the adenine of the urine. A similar increase in the purine output was not noticed when the other purines were fed. Brugsch and Schittenhelm,<sup>12</sup> in feeding purines to a gouty individual, found an increase of uric acid elimination of 50 per cent attributable to the adenine fed. Krüger and Solomon<sup>13</sup> have published an analysis of 10,000 liters of urine in which 10.11 grams of xanthine, 8.50 grams of hypoxanthine and 3.54 grams of adenine, were found. From the low result of adenine they conclude that much of the ingested adenine was destroyed. Jones<sup>14</sup> calls attention to the fact that by the method used (Neubauer's) hypoxanthine could be formed from adenine and carnine. Mendel and Lyman<sup>15</sup> found that the ingestion of adenine in man caused a marked rise in the uric acid elimination, and a small but noticeable increase in the elimination of purine bases. These results would seem to indicate that when adenine is fed to man some is destroyed, uric acid resulting as the end product in its metabolism, while a portion is not affected, but is excreted as the free base.

In view of these facts the following experiments were performed to ascertain whether adenase could be demonstrated *in vitro* in human tissues, either by examination of individual organs or of extracts of entire fetuses.<sup>16</sup>

#### EXPERIMENTAL PART.

In the following experiments given amounts of adenine or one of its salts were added to weighed samples of ground tissue with 500 cc. of water, and the mixtures incubated in air-tight bottles at 38° for two weeks, toluene being used as antiseptic. In all cases where sufficient tissue was available, control experiments were made, in which adenine was incubated with the extract of the same amount of tissue after the mixture had been boiled one hour. The procedure used for the isolation of purines at the end of that time was the copper sulphate and sodium bisulphite method of Krüger and Solomon, the adenine being determined as picrate and the hypoxanthine as hypoxanthine silver nitrate. In a previous set of purine analyses it was noted that certain precautions were necessary in the precipitation of adenine picrate.

<sup>12</sup> *Zeitschr. f. exp. Path. u. Ther.*, v, pp. 215-226, 1908.

<sup>13</sup> *Zeitschr. f. physiol. Chem.*, xxvi, p. 367, 1898.

<sup>14</sup> *Ibid.*, lxx, pp. 383-388, 1910.

<sup>15</sup> *This Journal*, viii, pp. 115-143, 1910.

<sup>16</sup> The materials used in this investigation were received from several Chicago Hospitals through the kindness of Drs. Jobling, Le Count, Gill, Henry and Davis.

Traces of mucilaginous substances from the tissue extracts may frequently be carried along in the analysis as far as the adenine stage, and these substances seem able to retard or even prevent the precipitation of adenine picrate. This difficulty can be overcome by reprecipitation of the purines with copper sulphate and sodium bisulphite, and treatment with picric acid of the cold filtrate after the decomposition of the copper purine compounds by hydrogen sulphide.

The liver, upon which the evidence in the literature with regard to the presence of adenase is contradictory, was first studied.

**EXPERIMENT I.** The liver used was from an accident case and was received a few hours after death. Microscopic examination showed it to be very fatty. 100 grams of the ground up tissue were added to a solution of 0.292 gram of adenine sulphate and the mixture kept, as in all the following experiments, at a temperature of 38°. It was very faintly acid, as were all the emulsions of this series of experiments. A boiled control, containing the same ingredients, was run at the same time. The figures for the purines added and those found by analysis after incubation, follow:

	ADDED		RECOVERED			
	Adenine sulphate	Calculated adenine	Adenine picrate	Calculated adenine	Hypo-xanthine Ag NO <sub>3</sub>	Calculated hypo-xanthine
Experiment.....	0.292	0.214	0.412	0.152	0.195	0.073
Control.....	0.292	0.214	0.442	0.164	0.124	0.046

**EXPERIMENT II.** The liver used, also from an accident case, was macroscopically and microscopically normal. 100 grams of tissue were used in the experiment and control. The results in this experiment, tabulated, were as follows:

	ADDED		RECOVERED			
	Adenine hydrochloride	Calculated adenine	Adenine picrate	Calculated adenine	Hypo-xanthine Ag NO <sub>3</sub>	Calculated hypo-xanthine
Experiment.....	0.230	0.170	0.294	0.109	0.194	0.073
Control.....	0.230	0.170	0.255	0.095	0.172	0.064

The adenine picrate separated out very slowly in this experiment even after reprecipitation of the purines by copper. Possibly a complete sep-



aration was not effected, in which case the hypoxanthine figures would be too high.

As other experiments have repeatedly shown that in the presence of adenase, adenine is completely converted into hypoxanthine in the amount used and time allowed above, and as in both cases the recovery of adenine (70 and 65 per cent) was about the same in the actual experiment as in the boiled controls, it may be concluded that the loss of adenine was due to experimental error and that adenase was not present. The hypoxanthine recovered represents the "preformed hypoxanthine" of the tissue used. The larger hypoxanthine recovery from the unboiled tissue can be explained as the result of the 14-day autolysis of the fresh tissue, it having been shown that in autolysis of tissues hypoxanthine is regularly formed even in the absence of adenase (see above and Amberg and Jones<sup>17</sup>).

As no work on the presence of adenase in the human placenta was found reported in the literature, the action of an extract of this organ upon adenine was studied:

EXPERIMENT III. (100 grams of tissue used.)

	ADDED		RECOVERED			
	Adenine sulphate	Calculated adenine	Adenine picrate	Calculated adenine	Hypoxanthine Ag NO <sub>3</sub>	Calculated hypoxanthine
Experiment.....	0.215	0.161	0.329	0.122	0.102	0.038
Control.....	0.215	0.161	0.391	0.145	0.046	0.017

The inability of extract of placenta to destroy adenine is thus shown. It may be mentioned that the action of the placental extract upon guanine was studied at the same time and the presence of guanase demonstrated by the complete conversion of added guanine into xanthine.

The following experiments were made either with the individual organs or with uniform samples of the ground up tissue of entire human fetuses. As mentioned above Wells and Corper<sup>18</sup> by the latter method obtained evidence of the presence of adenase in the bodies of fetuses above the fifth month.

<sup>17</sup> *Loc. cit.*

<sup>18</sup> *Loc. cit.*

EXPERIMENT IV. Male fetus, about the seventh or eighth month length 41 cm.; weight 1260 grams. Liver. (47.5 grams of tissue used.)

	ADDED		RECOVERED			
	Adenine sulphate	Calculated adenine	Adenine picrate	Calculated adenine	Hypo-xanthine Ag NO <sub>3</sub>	Calculated hypo-xanthine
Experiment.....	0.219	0.161	0.342	0.127	0.096	0.036

The rest of the fetus was ground up separately and the resulting emulsion thoroughly mixed. A 100-gram sample, representing all the organs of the body except the liver, was incubated for two weeks with a solution of 0.172 gram of adenine sulphate. A boiled control was run as usual. Very great difficulty and corresponding loss of purines due to the gelatinous material present were experienced in the filtration from tissue following the period of incubation. The use of aluminum cream and animal charcoal as coagulants was tried without much success. The mixtures were finally filtered on a hot funnel and the purine separation was made as usual. The solution representing the fresh tissue yielded no precipitate with picric acid on forty-eight hours' standing, while a precipitate of adenine picrate (m. p. 280°) came out readily in the boiled control. The figures for this experiment follow (100 grams of tissue were used):

	ADDED		RECOVERED			
	Adenine sulphate	Calculated adenine	Adenine picrate	Calculated adenine	Hypo-xanthine Ag NO <sub>3</sub>	Calculated hypo-xanthine
Experiment.....	0.172	0.126	0	0	0.123	0.045
Control.....	0.172	0.126	0.159	0.059	0.048	0.018

Adenase was evidently not present in the liver, adenine being recovered in quantity after treatment with the fresh tissue. The evidence with respect to the rest of the tissue is not conclusive. Adenase appeared to be present. Adenine was recovered from the boiled control, while no adenine could be recovered after treatment of its solution with 100 grams of the fresh tissue, but the quantity of hypoxanthine recovered was too small to be taken as representing with absolute certainty the action of adenase upon the adenine added, although the difficulties and consequent loss in analysis may account for the low recovery.

EXPERIMENT V. Female fetus about the fifth or sixth month; length 29 cm.; weight 490 grams. (The liver had been removed at the hospital.)

The body was ground up and uniformly mixed, and the action of a portion of the emulsion upon adenine tested as usual; 100 grams of tissue were used.

	ADDED		RECOVERED			
	Adenine hydrochloride	Calculated adenine	Adenine picrate	Calculated adenine	Hypoxanthine Ag NO <sub>3</sub>	Calculated hypoxanthine
Experiment.....	0.134	0.100	0.208	0.077	0.093	0.035
Control.....	0.134	0.100	0.251	0.093	0.036	0.013

As seen, adenine was recovered both from the experiment and from the boiled control.

**EXPERIMENT VI.** Female fetus; full term; length 52.7 cm.; weight 2190 grams. (The brain had been removed at the hospital.) The entire fetus was ground up and uniformly emulsionized as usual. 100 grams of tissue were used. The analytical data were:

	ADDED		RECOVERED			
	Adenine sulphate 2H <sub>2</sub> O	Calculated adenine	Adenine picrate	Calculated adenine	Hypoxanthine Ag NO <sub>3</sub>	Calculated hypoxanthine
Experiment.....	0.261	0.160	0.317	0.118	0.120	0.045
Control.....	0.261	0.160	0.329	0.123	0.041	0.015

Clearly the samples of tissue used in the last two experiments were unable to attack adenine, practically as much of the latter being recovered from the fresh extracts as from the boiled controls.

**EXPERIMENT VII.** Female fetus; full term; weight 3760 grams. The entire fetus was ground up and two uniform samples were taken as usual, and incubated with solutions each containing 0.123 gram of adenine, one of which was boiled for control. After fourteen days the purine separation was made as usual. No adenine could be recovered by picric acid precipitation in the solution representing the action of the fresh tissue, while adenine picrate settled out characteristically in the boiled control. The purines of this solution were then reprecipitated with copper sulphate and sodium bisulphite and the copper compounds decomposed as usual by hydrogen sulphide. Adenine could not be recovered from the clear, colorless filtrate from copper sulphide, even on treatment of the solution with picric acid over night. So hypoxanthine was then determined with certain precautions. The purines were reprecipitated as the copper compounds and then as the silver compounds. The silver nitrate was next made in the usual manner by treatment of the silver purine compound with hot dilute nitric acid. A few crystals of urea were added to prevent the deaminizing action of any trace of nitrous acid in the nitric acid used, which was of the purest variety, upon any adenine possibly present. The

crystalline compound which settled out upon cooling weighed 0.181 gram, corresponding to 0.068 gram of hypoxanthine. This yield was of course low and for the following reasons: the purification was quite complete and attended by the usual slight, unavoidable losses at each stage, and furthermore the substance was crystallized from a rather large volume of water (150 cc.). It was next recovered as the free purine and crystallized as the hydrochloride. The latter was prepared in the anhydrous form and analyzed for nitrogen by the Kjeldahl method.

0.0272 gram contained 0.0091 gram N or 33.45 per cent. Theory for hypoxanthine hydrochloride,  $C_5H_7N_4O \cdot HCl$ , 32.48 per cent. As anhydrous adenine hydrochloride contains 40.8 per cent N there can be no question as to the identity of the recovered compound.

From the boiled control adenine was recovered quantitatively as shown in the following tabulation of the results of this experiment. 100 grams of tissue were used.

	ADDED		RECOVERED		
	Adenine	Adenine picrate	Calculated adenine	Hypoxanthine $AgNO_3$	Calculated hypoxanthine
Experiment.....	0.123	0	0	0.181	0.068
Control.....	0.123	0.322	0.119	0.056	0.021

The presence of adenase in the fetus used in this case seems to be demonstrated. Adenine was recovered quantitatively from a boiled control, while it could not be recovered at all from the fresh extract, its place being taken by hypoxanthine.

The finding of the enzyme in an extract of fetal tissue taken into consideration with the negative findings of Jones and his coworkers in the study of adult organs, suggested the possibility that adenase was present in the organ, well developed in the infant but atrophied in the adult, viz., the thymus. As mentioned above, Schittenhelm and Schmid<sup>19</sup> reported the destruction of adenine by extracts of the human thymus. Accordingly this organ was examined for adenase, with negative result:

EXPERIMENT VIII. (2.5 grams thymus tissue used.)

ADDED			RECOVERED		
Adenine sulphate	Calculated adenine	Adenine picrate	Calculated adenine	Hypoxanthine $AgNO_3$	Calculated hypoxanthine
0.130	0.098	0.198	0.078	0.010	0.004

<sup>19</sup> *Loc. cit.*

Thus adenase was not present. The result of Schittenhelm and Schmid was not confirmed.

Other fetuses were next obtained and after the removal of some of the organs for individual study, examined in the same manner as the preceding ones.

EXPERIMENT IX. Female fetus, about seventh month; length 38 cm.; weight 1140 grams.

*Brain.* (50 grams of tissue were used.)

	ADDED		RECOVERED			
	Adenine sulphate	Calculated adenine	Adenine picrate	Calculated adenine	Hypo-xanthine Ag NO <sub>3</sub>	Calculated hypo-xanthine
Experiment.....	0.199	0.145	0.367	0.136	0.035	0.013
Control.....	0.199	0.145	0.362	0.134	0.006	0.002

*Bones.* (50 grams, ground and mixed.)

	ADDED		RECOVERED			
	Adenine sulphate	Calculated adenine	Adenine picrate	Calculated adenine	Hypo-xanthine Ag NO <sub>3</sub>	Calculated hypo-xanthine
Experiment.....	0.199	0.145	0.242	0.090	0.114	0.043
Control.....	0.199	0.145	0.335	0.124	0.008	0.003

The other tissues were ground up and uniformly mixed. 100-gram samples of the uniform emulsion were taken as in the preceding experiments.

	ADDED		RECOVERED			
	Adenine sulphate	Calculated adenine	Adenine picrate	Calculated adenine	Hypo-xanthine Ag NO <sub>3</sub>	Calculated hypo-xanthine
Experiment.....	0.199	0.145	0	0	0.324	0.123
Control.....	0.199	0.145	0.236	0.088	0.086	0.032

Adenase was clearly absent from the brain and bone, adenine being recovered in quantity after treatment with the extract of each tissue. It appeared to be present however in the mixed mass of ground tissue representing all the other organs of the body. No adenine could be recovered after treatment with this tissue, its place being taken by hypoxanthine, while it was recovered in considerable quantity from the boiled control.

EXPERIMENT X. Male fetus; seventh to eighth month; length 40 cm.; weight, 1670 grams. The stomach, intestines, and pancreas, were removed, ground together, and tested for adenase in the usual manner. The results were as follows:

	ADDED		RECOVERED*			
	Adenine sulphate	Calculated adenine	Adenine picrate	Calculated adenine	Hypoxanthine Ag NO <sub>3</sub>	Calculated hypoxanthine
Experiment.....	0.147	0.107	0.184	0.068	0.036	0.014
Control.....	0.147	0.107	0.164	0.061	0.036	0.014

\*An accident in manipulation lowered the recovery by about a fourth in each case.

All the remaining tissues were ground together and intimately mixed. Uniform samples of 100 grams were taken as usual:

	ADDED		RECOVERED			
	Adenine sulphate	Calculated adenine	Adenine picrate	Calculated adenine	Hypoxanthine Ag NO <sub>3</sub>	Calculated hypoxanthine
Experiment.....	0.147	0.107	0	0	0.306	0.115
Control.....	0.147	0.107	0.134	0.050	0.191	0.071

Apparently adenase was present in an emulsion of all the tissues other than the stomach, intestines, and pancreas—it could not be demonstrated in the latter tissues—for hypoxanthine and no adenine was recovered after treatment with the fresh tissue. The results are, however, not conclusive, for unfortunately a slight putrefactive odor was noted in the mixture after incubation, showing that some bacterial action had taken place in spite of the antiseptic added, so that the possibility of conversion by bacterial enzymes cannot be excluded. The recovery of adenine from the boiled control was for some unknown reason lower than usual.

The hypoxanthine fractions recovered from the tests with the entire fetuses in Experiments IX and X were put together, repurified, and the purine crystallized as the anhydrous hydrochloride.

Analysis (Kjeldahl).....32.71 per cent N.  
 Theory for hypoxanthine hydrochloride,  
 $C_5H_7N_4O \cdot HCl$ .....32.48 per cent N.

EXPERIMENT XI. Male fetus; full term; weight 3665 grams. The lungs, intestines, and spleen, were examined separately, with the results tabulated below:

*Lungs.* (40 grams of tissue used.)

ADDED	RECOVERED			
Adenine	Adenine picrate	Calculated adenine	Hypoxanthine Ag NO <sub>3</sub>	Calculated hypoxanthine
0.197	0.489	0.181	0.099	0.037

*Intestines.* (50 grams of tissue used.)

ADDED	RECOVERED			
Adenine	Adenine picrate	Calculated adenine	Hypoxanthine Ag NO <sub>3</sub>	Calculated hypoxanthine
0.197	0.465	0.172	0.058	0.023

*Spleen.* (10 grams of tissue used.)

ADDED	RECOVERED			
Adenine	Adenine picrate	Calculated adenine	Hypoxanthine Ag NO <sub>3</sub>	Calculated hypoxanthine
0.123	0.306	0.113	0.020	0.007

An emulsion was also made containing portions of all the other tissues of the body, which was examined for the presence of adenase in the usual manner: 100 grams of tissue were used.

	ADDED	RECOVERED			
	Adenine	Adenine picrate	Calculated adenine	Hypoxanthine Ag NO <sub>3</sub>	Calculated hypoxanthine
Experiment.....	0.197	0.347	0.139	0.273	0.102
Control.....	0.197	0.542	0.200	0.061	0.024

Thus adenase was found to be absent from the lungs, intestines, and spleen, and its presence could not be demonstrated in an emulsion of the other tissues, added adenine being recovered in all cases. The amount of adenine recovered from the treatment with the emulsion was lower than that recovered from the boiled control and the hypoxanthine recovery was considerably higher.

However, this cannot safely be interpreted as a result of the action of adenase for the losses due to mechanical factors may show considerable variance in operations involving the use of the quantity of tissue here employed, and a high hypoxanthine recovery is a finding which was regularly observed in this series of experiments where 100 grams of tissue were allowed to autolyze for two weeks. This formation of hypoxanthine in the autolysis of tissues not containing adenase, as mentioned before, is explained by Amberg and Jones<sup>20</sup> as the result of a transformation of the adenine radicle of nucleic acid by way of other intermediate products than adenine, viz., adenosine and inosine, adenosine deaminase and inosine hydrolase being the enzymes concerned in the process.

Adenase was clearly absent from the lungs, intestines, and spleen, adenine being recovered quantitatively after treatment with the fresh tissue, a result in agreement with the findings of Miller and Jones.<sup>21</sup> The results obtained with the mixed tissues of the fetuses used in this investigation are, as seen, inconsistent. In Experiments IV, VII, IX, and X adenine added to an extract of the fresh thoroughly mixed tissue representing portions of the entire body other than those taken for separate examination, could not be recovered after two weeks' incubation, hypoxanthine being found in its place. These findings are in agreement with those of Wells and Corper<sup>22</sup> in the case of fetuses of the five and six month stages. The negative result in Experiment V in which a five-month fetus was used may possibly be explained on the assumption that the enzyme had not yet developed. The experiments of Jones and Austrian,<sup>23</sup> Wells and Corper<sup>24</sup> and Mendel and Mitchell<sup>25</sup> show that the purine enzymes appear at different periods and it is reasonable to suppose that under certain conditions the appearance might be delayed. But the results of Experiments VI and XI in which the enzyme could not be demonstrated in the bodies of full term fetuses, seem hardly reconcilable.

<sup>20</sup> *Loc. cit.*

<sup>21</sup> *Loc. cit.*

<sup>22</sup> *Loc. cit.*

<sup>23</sup> This *Journal*, iii, pp. 227-232, 1907.

<sup>24</sup> *Loc. cit.*

<sup>25</sup> *Amer. Journ. of Physiol.*, xx, pp. 81-96, 1907.



with those of Experiments IV, VII, IX, and X in which extracts of emulsionized fetuses converted adenine into hypoxanthine. (The result in Experiment X is open to question as explained in connection with that experiment.) It would seem that adenase is contained in the body of the human fetus, but that its location is not known, and whether because of its low concentration or for some other reason uniform samples from some emulsionized bodies may show its presence while others may not.

#### SUMMARY.

Adenase could not be demonstrated in the human adult liver, the placenta, or the fetal liver, brain, bone, thymus, stomach, intestines, pancreas, lungs, and spleen.

Evidence of its presence in the body of the human fetus was obtained, however, by treating adenine with 100-gram samples of thoroughly mixed tissues of the entire bodies in the case of four fetuses, while such evidence was lacking in the case of three other fetuses examined.

Hypoxanthine is always formed in the autolysis of human tissues, whether they contain adenase or not, a finding which signifies a transformation of the adenine radicle of nucleic acid by way of other intermediate products than adenine, probably through the action of adenosine deaminase and inosine hydrolase, as suggested in the case of similar findings in other tissues by Amberg and Jones.



### GLYOXALASE. PART III.

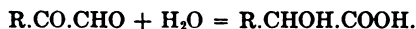
#### THE DISTRIBUTION OF THE ENZYME AND ITS RELATION TO THE PANCREAS.

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In two previous communications we have dealt with the preparation, mode of action and conditions for optimum activity of glyoxalase.<sup>1</sup> This enzyme converts  $\alpha$ -ketonic aldehydes such as methyl and phenyl glyoxals into optically active lactic and mandelic acids:



We have already referred to the possible physiological significance in connection with the metabolism of carbohydrates.<sup>2</sup> In the present communication we wish to deal further with the distribution of the enzyme and particularly with the effect of the pancreas in inhibiting its action.

We have found no difficulty in obtaining highly active glyoxalase preparations from the blood, liver or muscle of a variety of animals, including man, dog, cat, calf, sheep, rabbit, fowl, toad, codfish, skate and oyster. On the other hand vegetable organisms, such as yeast and green plants, contain relatively less glyoxalase, and in some cases our experiments with plant material have given entirely negative results. In general it may be said that the distribution of the enzyme harmonizes with its supposed function.

Extracts of all animal tissues that we have examined have given definitely positive indications of the presence of glyoxalase with one exception, namely, the pancreas. Since blood cells contain much glyoxalase the positive findings in the case of some organs

<sup>1</sup> This *Journal*, xiv, pp. 155, and 423, 1913.

<sup>2</sup> *Ibid.*, xiv, p. 555, 1913.

may possibly be due to unavoidable contamination but in most cases the glyoxalase is certainly present in the tissue cells. Glyoxalase is absent from urine and bile. *Further experiments showed that pancreatic tissue, aqueous extracts of the pancreas and pancreatic juice not only contain no glyoxalase but contain a thermolabile substance which exerts an intense inhibitory action on glyoxalase derived from other sources.*

We have observed the inhibitory action of the pancreas on glyoxalase in every animal species that we have examined, including man, dog, cat, rabbit, fowl and toad. In view of the known connection of the pancreas with carbohydrate metabolism it appeared desirable to study the relation between the pancreas and glyoxalase in some detail. The results which we have obtained thus far may be summarized as follows:

1. The inhibitory action of the pancreas of one species of animal is not limited to glyoxalase derived from the same species.

2. The inhibitory substance, which for convenience we may call antiglyoxalase, is found not only in extracts of the gland but is present in pancreatic juice obtained from dogs either by fistula or as the result of the injection of secretin. It is present in commercial trypsin and pancreatin preparations and may be preserved in the dry state indefinitely. A pancreas preparation more than eight years old was found to contain much antiglyoxalase.

3. Antiglyoxalase is destroyed by heating for ten minutes at 85°. It is slowly destroyed by digestion with weak hydrochloric acid ( $\frac{N}{10}$ ) much as trypsin is. The action of weak alkali (1 per cent sodium carbonate) is much less injurious.

4. Relatively small amounts of pancreas extract can inhibit the action of large amounts of active glyoxalase. Thus 0.025 gram of a pancreatin preparation containing much inert material or less than 2 cc. of pancreatic juice may completely inhibit the action of a glyoxalase solution which otherwise would be capable of forming several grams of lactic acid.

5. By observing the effect of varying amounts of antiglyoxalase during different intervals of time upon glyoxalase we have found that the inactivation of the latter is not instantaneous but is a function of the time. It is possible that antiglyoxalase is an enzyme accelerating the normal decomposition of glyoxalase and all the facts are in agreement with this hypothesis, but they could

be equally well reconciled with the view that the inactivation of glyoxalase is brought about by simple direct union with antiglyoxalase without catalytic action. We have been unable as yet to settle this question definitely.

6. The inhibitory action of the pancreas upon glyoxalase is not due to trypsin, lipase or diastase. An antiglyoxalase solution may be freed from trypsin by digestion with sodium carbonate and the resulting solution which is entirely without digestive action on proteins may still possess its inhibitory action upon glyoxalase. Moreover the vegetable proteoclastic enzyme papayin is entirely without action upon glyoxalase. Neither lipase nor diastase apparently are responsible for the action of antiglyoxalase, since liver extracts containing much lipase, and salivary diastase have no action upon glyoxalase.

7. Saliva, extracts of gastric mucosa from the middle and cardiac end of the stomach, blood serum or small amounts of bile are without action on glyoxalase. Extracts of duodenal mucosa on the other hand have a pronounced inhibitory effect, but it is probable that this is due to antiglyoxalase of pancreatic origin.

It is noteworthy that the so-called "pyloric caeca" of the cod-fish which were long supposed to be homologous with the pancreas not only contain no antiglyoxalase but contain a small amount of glyoxalase. It is now generally recognized by biologists that the pyloric caeca have nothing to do with the pancreas which in the adult is extremely inconspicuous and liable to escape observation. It is interesting that the chemical and morphological findings should harmonize so satisfactorily.<sup>3</sup>

While the pancreas contains no glyoxalase we find that the thyroid of the dog yields a very active preparation. We propose examining the pituitary and suprarenal glands as soon as we are able to secure suitable material.

In view of the relation existing between the pancreas and glyoxalase, and the function of both in connection with carbohydrate metabolism it appeared desirable to examine tissues of diabetic and other pathological organisms with regard to their glyoxalase. We find that glyoxalase is present in human blood from diabetics and in blood and liver from diabetic dogs which have had their

<sup>3</sup> Professor E. B. Wilson was kind enough to give us advice upon the biological matters.

pancreas removed ten days previously. Thus far our results appear to show that while the glyoxalase of the blood of glycosuric dogs is not changed from normal, the blood and tissues of human diabetics and of depancreatized dogs have somewhat less than the normal activity. It is curious that the removal from an animal of the organ which exerts so profound an inhibitory effect upon the action of glyoxalase should be followed by a decrease rather than an increase in the activity of the latter enzyme. It would be possible to indulge in many speculations as to the relation of glyoxalase to pancreatic diabetes. But the conditions especially in the case of these experiments with pathological material are very complicated and before we can draw any satisfactory conclusions it will be necessary to study the problem with better methods than we have at present been able to apply. We hope to return to these experiments later.

The opportunity for making the experiments with depancreatized dogs as well as an experiment with pancreatic juice and a number of others that are not reported in this paper, was afforded us by the kindly coöperation of Professors Richards and Sweet of the University of Pennsylvania. We wish to record our grateful appreciation of their generous assistance.

#### EXPERIMENTAL.

In all the experiments recorded in this paper we have made use of phenyl glyoxal as substrate rather than methyl glyoxal. We have satisfied ourselves that the action of glyoxalase on the two glyoxals is essentially similar, but the use of phenyl glyoxal has certain important advantages. In the first place the mandelic acid which is formed from it, is easily extracted by ether and crystallizes readily. In every case in which we record a positive glyoxalase reaction the mandelic acid has been isolated in clean crystalline form. The high optical activity of mandelic acid as compared with the low rotation of lactic acid is also a great advantage. For each separate digestion we have used 0.2 gram of phenyl glyoxal.

The tissue extracts were in almost all cases prepared by stirring one part of minced tissue with five parts of water and straining through muslin after one hour. The use of plain water rather than saline is a distinct improvement since it effects a much

better extraction of the mass and yields solutions with greater enzyme activity. Blood cells, leucocytes, etc., should not be washed with saline as much glyoxalase is lost in the process.

In most of the experiments we have avoided the use of toluene or have used it in limited amount since it has a decidedly injurious effect upon glyoxalase (Section V). By using sterile tissues taken directly from the animal the use of antiseptics is unnecessary. Moderate bacterial contamination such as may occur after short periods of digestion is entirely without effect on the action of glyoxalase and moreover the addition of phenyl glyoxal appears to favor asepsis. In every case precipitated chalk was used to maintain approximate neutrality.

The digestion mixtures were analyzed precisely as described in our former paper<sup>4</sup> except that the heating in the water bath was limited to three minutes and the mandelic acid was extracted by shaking with four successive quantities of ether. The residual mandelic acid crystals were dissolved in 10 cc. of water, treated with a pinch of charcoal if necessary and then examined in a 2 dm. tube in the polarimeter. Subsequently the acidity was determined by titrating 5 cc. with decinormal caustic soda. In the following records of experiments the rotations given are those actually observed in the 2 dm. tube, while the acidities represent the number of cubic centimeters of alkali required to neutralize the 10 cc. of mandelic acid solution. In general it is advisable to judge of the enzyme activity rather by the rotation of the mandelic acid than by the acidity since the organ extracts themselves yield relatively considerable amounts of acid so that the observed acidities are always too high. Special blank experiments have repeatedly shown that under the existing conditions no optically active substance other than mandelic acid occurs in the extracts in sufficient amount to give a significant rotation.

#### *I. Distribution of glyoxalase.*

In each experiment, unless otherwise stated, 50 cc. of strained 20 per cent tissue extract or 5 cc. of blood were allowed to act on 0.2 gram of phenyl glyoxal in the presence of chalk for about 20 hours.

<sup>4</sup> This *Journal*, xiv, p. 427, 1913.

ANIMAL	TISSUE	ACIDITY	ROTATION OF MANDELIC ACID	PRES- ENCE OF GLYOKA- LASE
		cc.	deg. C	
Man	Blood.....	2.3 to 3.8	-1.03 to -1.30	+
	Pancreas.....	0.5	-0.02	-
	Urine (150 cc.).....	2.2	0	-
	Muscle.....	3.0 to 3.4	-0.45 to -1.13	+
	Liver.....	6.0 to 8.8	-2.3 to -2.58	+
	Heart muscle.....	already reported		+
	Kidney.....	already reported		+
	Brain.....	already reported		+
	Lung.....	already reported		+
Dog	Thyroid.....	8.2	-1.65	+
	Gastric mucosa.....	4.7	-0.95	+
	Duodenal mucosa.....	1.3	-0.12	?
	Blood.....	3.6	-0.68 to -1.35	+
	Blood serum.....	1.4	0 to +0.05	-
	Bile.....	1.2	0	-
	Pancreas.....	1.4 to 2.0	0 to +0.05	-
	Pancreatic juice (20 cc.).....	1.2	-0.03	-
	Liver.....	7.2	-2.02	+
Calf	Pancreas.....	1.2	+0.05	-
	Bile.....	1.3	+0.03	-
Sheep	Blood.....	3.2	-0.83	+
	Muscle.....	6.6	-2.22	+
Cat	Liver.....	8.3	-2.60	+
	Blood.....	1.9	-0.33	+
	Pancreas.....	1.2	-0.08	-
Rabbit	Liver, muscle.....	8.4	-2.40	+
	Blood.....	3.0	-0.75	+
	Liver.....	7.6	-2.51	+
Fowl	Muscle.....	6.9	-2.80	+
	Blood.....	5.4	-2.42	+
	Pancreas.....	1.6	-0.05	-
Codfish	Liver.....	1.6	-0.35	+
	Muscle.....	1.0	-0.20	+
	Pyloric caeca.....	1.4	-0.17	+
Skate	Muscle.....	2.6	-0.83	+
Toad	Liver.....	8.0	-2.57	+

## II. The effect of adding extracts of pancreas on the action of glyoxalase.

The experiments recorded in this section were made essentially in the same manner as those in Section I. Only a few of the experiments which were carried out are recorded as they all gave



substantially similar results. A variety of pancreatic extracts and commercial pancreatin preparations were used without disclosing any marked differences. The column marked "time" in the following table indicates the time during which the added pancreas preparation was allowed to act on the glyoxalase before adding phenyl glyoxal. Where no entry is made it is to be understood that phenyl glyoxal was added immediately. In each case 50 cc. of glyoxalase solution, 0.2 gram phenyl glyoxal, and excess of precipitated chalk were used.

ANIMAL	TISSUE	ADDED SUBSTANCE	TIME	ROTATION OF MAN- DELIC ACID	CONCLUSION
			<i>hours</i>	<i>deg. C</i>	
Dog...	Muscle...	—	—	-1.07	+
		Pancreas, 50 cc.	—	+0.05	Inhibition
		Pancreas, 50 cc.	—	-0.13	Inhibition
Sheep...	Blood...	—	—	-0.83	+
		Ox pancreas, 100 cc.	—	+0.03	Inhibition
		—	—	-2.8	+
Fowl...	Muscle and Liver	Fowl pancreas, 0.75 gram	1	-1.88	Partial inhibition
		Fowl pancreas, 0.75 gram heated to 85°	1	-2.65	No inhibition
		Pancreatin, 0.2 gram	1	-0.08	Inhibition
		Pancreatin, 0.05 gram	1	-1.55	Partial inhibition
		Pancreatin, 0.025 gram	1	-1.88	Partial inhibition
		Pancreatin, 0.2 gram heated to 85°		-2.75	No inhibition
		—	—	-2.6	+
		Pancreatin, 0.2 gram	1	-0.32	Partial inhibition
Cat....	Muscle and Liver	Cat pancreas, 3 grams	1	-0.08	Inhibition
		—	—	-2.57	+
Toad...	Liver....	Toad pancreas, 0.5 gram	3	-0.40	Partial inhibition

*III. The action of pancreatic juice on glyoxalase.*

Two sets of experiments with pancreatic juice were made. In the first of these it was found that as little as 2 cc. of pancreatic juice obtained by secretin injection when acting for two hours on a glyoxalase solution completely inhibited the action of the enzyme. In the second experiment the juice which was obtained from a fistula was added to the glyoxalase simultaneously with the phenyl glyoxal. In this case the addition of a larger amount was necessary to secure inhibition. Heated juice was without adverse effect. Fifty cc. of glyoxalase solution, 0.2 gram of phenyl glyoxal, and excess of chalk were used in each experiment.

SOURCE OF GLYOXALASE	PANCREATIC JUICE	TIME BEFORE ADDING PHENYL GLYOXAL	ROTATION OF MANDELIC ACID	EFFECT
	cc.	hours	deg. C	
Heart and tongue of dog.....	—	—	-0.45	
	2	2	-0.07	Inhibition
	5	2	-0.03	Inhibition
	10	2	+0.05	Inhibition
Skeletal muscle of dog.....	—	—	-1.13	
	2	—	-1.28	No inhibition
	20	—	-0.25	Marked inhibition
	2	—	—	
	(heated to 85°)	—	-1.38	No inhibition
	20 (heated to 85°)	—	-1.68	No inhibition

*IV. The inactivation of glyoxalase by small amounts of antiglyoxalase acting for varying lengths of time.*

Experiments were made to determine the rate of inactivation of glyoxalase by small amounts of antiglyoxalase with a view to gaining insight into the nature of the inhibitory substance. A small quantity of "pancreatin," suspended in a known volume of water, was added to a 20 per cent glyoxalase extract. The mixture was then divided into a number of equal parts which were placed in flasks containing chalk suspension. To one of these solutions phenyl glyoxal was immediately added and all were placed in the incubator at 37°C. After suitable periods

phenyl glyoxal was added to the contents of the other flasks, so that eventually a series of experiments was obtained in which the relative activities of equal amounts of glyoxalase after varying periods of incubation with identical quantities of antiglyoxalase were determined. Control experiments were made by following simultaneously the loss of activity of corresponding amounts of the original glyoxalase extract at incubator temperature.

In Experiment I, five flasks, each containing 50 cc. of 20 per cent glyoxalase extract from dog's liver, 0.05 gram of pancreatin and precipitated chalk were prepared. To one of these 0.2 gram of phenyl glyoxal was added immediately and all were placed in the incubator at 35°C. After one, three, five and seven hours a similar quantity of phenyl glyoxal was added to each of the other flasks respectively. Control experiments with 50 cc. of liver extract, fresh and after incubation for seven hours, were made.

Experiment II was carried out similarly to Experiment I. A 20 per cent extract of cat's muscle and liver was employed, and to each 50 cc. of this preparation 0.035 gram of "pancreatin" was used. Phenyl glyoxal (0.2 gram) was added at the beginning and after every hour for six hours. Three control experiments were made with fresh and incubated glyoxalase extract (50 cc.).

All solutions were incubated for about twenty hours after the addition of phenyl glyoxal and then worked up for mandelic acid in the usual way.

The following table gives the results of the two experiments.

EXP.	TIME	ROTATION OF MANDELIC ACID FROM	
		Glyoxalase-pancreatin mixture	Glyoxalase extract
	hours	deg. C	deg. C
I.....	0	-2.38	-2.58
	1	-1.9	
	3	-0.45	
	5	0	
	7	0	-1.15
II.....	0	-1.63	-2.22
	1	-1.27	
	2	-0.47	
	3	-0.17	-1.73
	4	-0.10	
	5	0	
	6	+0.05	-1.3

The mandelic acid residues were dissolved in 10 cc. of water and examined in a 2 dm. tube. The rotations are those actually observed. The time of incubation before the addition of phenyl glyoxal is recorded.

*V. The effect of adding substances other than pancreas upon the action of glyoxalase.*

These experiments were carried out in precisely the same manner as those in Section II. They show that saliva, gastric mucosa, blood serum and digestion with papayin have no effect upon glyoxalase. In two experiments with bile no inhibition was noted, in the third a marked decrease in the action of glyoxalase was noted, but we are inclined to attribute this to precipitation caused by the very concentrated bile interfering with the action of the

SOURCE OF GLYOXALASE	ADDITION	TIME BEFORE ADDING PHENYL GLYOXAL	ROTATION OF MANDelic ACID	CONCLUSION
		hours	degrees	
Fowl.....	—	—	-2.88	
	10 cc. Saliva	3	-2.98	No inhibition
	—	—	-0.45	
Dog.....	Gastric mucosa, 5 grams	3	-1.75	No inhibition
	—	—	-2.30	
	Duodenal mucosa, 40 cc.	3	-0.33	Partial inhibition
Cat.....	—	—	-2.6	
	Duodenal mucosa, 37 cc.	1	-0.45	Partial inhibition
	—	—	-2.30	
Dog.....	Dog bile, 2 cc.	3	-2.28	No inhibition
	Dog bile, 3 cc.	3	-2.25	No inhibition
Cat.....	Cat bile, 3 cc.	3	-0.13	Inhibition (?)
	—	—	-2.20	
Fowl.....	Papayin, 0.2 gram	1	-2.57	No inhibition
	Papayin, 0.2 gram	24	-2.45	No inhibition
	—	—	-1.35	
Dog.....	Dog serum, 20 cc.	—	-1.44	No inhibition
	Toluene, 2 cc.	2½	-0.08	Marked inhibition
	Toluene, 2 cc.	3	-0.12	Marked inhibition

chalk used for neutralization. The inhibitory action of duodenal mucosa extracts and the destruction of glyoxalase by toluene are clearly shown. In all cases the glyoxalase was obtained from the liver and skeletal muscles of the animals indicated except in the experiments with toluene when blood was used.

#### VI. *The separation of antiglyoxalase from trypsin.*

Experiments were made to remove trypsin without destroying antiglyoxalase by digesting an active pancreatin preparation with dilute acid or alkali. The experiments with alkali were successful while digestion with acid ( $\frac{N}{100}$  hydrochloric acid) gave less favorable results since the antiglyoxalase was partly destroyed before all the trypsin was removed.

Pancreatin (0.2 gram) containing much antiglyoxalase was digested with 10 cc. of 1 per cent sodium carbonate solution at 37° for four, twenty and twenty-four hours respectively. After four hours' digestion the pancreatin had very little or no action on coagulated egg white and the trypsin was entirely destroyed with certainty in the case of the longer digestions. Each pancreatin preparation was added to 50 cc. of an active glyoxalase solution and digested for three hours, when 0.2 gram phenyl glyoxal and precipitated chalk were added and the mixtures incubated for twenty-four hours. The rotations (2 dm. tube) of the mandelic acid extract (10 cc.) were as follows:

(1) Glyoxalase + no pancreatin.....	-2.22°
(2) Glyoxalase + pancreatin digested 4 hours.....	-0.05°
(3) Glyoxalase + pancreatin digested 20 hours.....	+0.03°
(1) Glyoxalase + no pancreatin.....	-3.05°
(2) Glyoxalase + pancreatin digested 24 hours.....	-0.73°

The results indicate clearly that trypsin may be removed from pancreatin without destroying the action of antiglyoxalase.

#### VII. *Glyoxalase in diabetic tissues.*

These experiments are fragmentary and can only be regarded as preliminary to a more extended study. The human material was obtained for us by Dr. N. W. Janney of the Montefiore Home while, as already mentioned, we owe the material from depan-

creatized dogs to the kindness of Professors Richards and Sweet. The human diabetic blood was obtained from only moderately severe cases. The liver and pancreas were obtained at post mortem about twenty-four hours after death. The absence of glyoxalase from the liver cannot be regarded as significant although the enzyme does not usually disappear after death with great rapidity. The blood and liver from depancreatized dogs were obtained from nine to ten days after operation by Professor Sweet. In each case 5 cc. of blood or 50 cc. of 20 per cent tissue extract and 0.2 gram of phenyl glyoxal were used. The mixtures were analyzed as in previous experiments.

TISSUE	ACIDITY	ROTATION OF MANDELIC ACID <i>deg. C</i>	GLYOXALASE
Diabetic human blood....	2.2	-0.50	+
Diabetic human blood....	2.8	-0.92	+
Diabetic human blood....	2.6	-0.83	+
Diabetic human liver....	2.2	+0.08	-
Diabetic human pancreas	0.5	-0.02	-
Depancreatized dog blood	1.7	-0.45	+
Depancreatized dog liver..	4.6	-0.93	+
Phlorhizin dog blood....	3.2	-1.17	+

## ON THE ACTION OF LEUCOCYTES AND OTHER TISSUES ON *dl*-ALANINE.

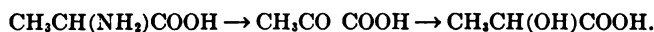
BY P. A. LEVENE AND G. M. MEYER.

(From the Rockefeller Institute for Medical Research, New York.)

(Received for publication, July 10, 1913.)

The observations on the action of leucocytes and other tissues under aseptic conditions on hexoses made it possible to disclose the mechanisms of lactic acid formations from glucose.<sup>1</sup> The fact that *d*-lactic acid was formed regardless of the configuration of the hexose employed in the experiments forced the conclusion that methyl glyoxal was the immediate precursor of lactic acid.

It is generally accepted principally on the basis of the work of Otto Neubauer<sup>2</sup> that the conversion of amino-acids into hydroxy-acids is brought about by a process very analogous to that for formation of lactic acid from methyl glyoxal. Thus, for instance, alanine is transformed into lactic acid by passing through the stage of pyruvic acid.



This contention found support in the observations of Lusk and Ringer.<sup>3</sup> These authors fed phlorhizinized dogs on *dl*-alanine and noted a complete conversion of the substance into *d*-glucose. Very recently this view was accepted on theoretical considerations by Dakin and Dudley.<sup>4</sup> However, in the attempt to corroborate this view experimentally not all writers came to a close agreement. For instance, Paul Mayer,<sup>5</sup> feeding phlorhizinized dogs on pyruvic acid failed to note a conversion of the substance into glucose. On the other hand, Dakin and Dudley, and Ringer,<sup>6</sup> obtained a positive result. However, they noted that quantitatively the yield of sugar from pyruvic acid was smaller

<sup>1</sup> Levene and Meyer: this *Journal*, xiv, pp. 149, 551, 1913.

<sup>2</sup> Neubauer: *Deutsch. Arch. f. klin. Med.*, xcv, p. 211, 1909.

<sup>3</sup> Ringer and Lusk: *Zeitschr. f. physiol. Chem.*, lxvi, p. 106, 1910.

<sup>4</sup> Dakin and Dudley: this *Journal*, xv, p. 127, 1913.

<sup>5</sup> Mayer: *Biochem. Zeitschr.*, xlix, p. 486, 1913.

<sup>6</sup> Ringer: this *Journal*, xv, p. 145, 1913.

than that from either alanine or from lactic acid. Hence, it was desirable if possible to find a simpler method of testing the theory of the mechanism of lactic acid formation from alanine. Encouraged by the results of the experiments on the action of leucocytes on glucose,<sup>7</sup> we attempted to test the action of leucocytes on the various optical isomers of alanine. It was thought that if pyruvic acid is the precursor of lactic acid then the same *d*-lactic acid might be obtained regardless of the nature of the alanine employed in the experiment. It was taken for granted that leucocytes possessed the power to deaminize alanine since it is generally accepted that the power of deaminization of amino-acids belongs to all tissues. The experiment was begun by the employment of *dl*-alanine. It was a surprise to find that under aseptic conditions leucocytes remained without perceptible action on *dl*-alanine. An attempt was therefore made to test the action of other tissues under aseptic conditions on *dl*-alanine. For this purpose the kidney was employed as previous experience had shown that the kidneys were the most suitable material that could be obtained in absolutely aseptic condition. However, the results of the latter experiments were identical with the results of the leucocyte experiments, namely, they showed that no perceptible deaminization took place.

Since these two series of experiments seemed not to harmonize with the generally accepted view on the deaminizing action of the tissues on amino-acids, it was thought that a considerable degree of autolysis of the tissues is required in order to bring their deaminizing action into play. For this reason experiments were performed with tissues in the presence of antiseptics. Kidney and liver were used in the experiments. However, when sufficient antiseptic was added to entirely prevent bacterial contamination no evidence was found of the deaminization of alanine in experiments that lasted from one to three weeks.

It is seen from this that alanine is not so readily deaminized as one was inclined to expect on the basis of the work of Lang<sup>8</sup> and other observers. Whether or not this is true in regard to other amino-acids remains to be established. In a recent article on deaminization by Gertrude D. Bostock,<sup>9</sup> from Cathcart's labo-

<sup>7</sup> Levene and Meyer: this *Journal*, xi, p. 361, 1912; xii, p. 265, 1912.

<sup>8</sup> Lang: *Hofmeister's Beiträge*, v, p. 321, 1904.

<sup>9</sup> Bostock: *Biochem. Journ.*, vi, p. 48, 1912.



ratory, a statement is made that alanine behaves differently from glycocoll on treatment with liver and intestinal mucosa, but the alanine experiments are not reported in detail. We hope to repeat our observations on alanine on a series of other amino-acids.

#### EXPERIMENTAL.

*Tissues.* The kidney and liver used in these experiments were obtained from dogs which had been killed by exsanguination from the carotid while in ether narcosis. The tissues were hashed in a meat chopper and immediately weighed and minced with the alanine and phosphate solutions as described below. The leucocytes were obtained aseptically from the pleural cavity of dogs injected with turpentine according to the method previously described.<sup>10</sup> In one experiment the kidneys of a rabbit removed aseptically were used.

*Solutions.* *dl*-Alanine in approximately 2 per cent concentration in a 1 per cent Henderson phosphate mixture was used in all experiments, excepting the leucocyte and aseptic kidney experiment. Toluene was used as antiseptic. The mixtures for each experiment were made up as follows:

##### *Kidney.*

1. 20.0 grams tissue suspended in 200 cc. alanine solution.
2. 5.0 grams tissue suspended in 50 cc. alanine solution.
3. 5.0 grams tissue suspended in 50 cc. phosphate solution.
4. 5.0 grams tissue suspended in 50 cc. phosphate solution.

##### *Liver.*

1. 40.0 grams tissue suspended in 200 cc. alanine solution.
2. 10.0 grams tissue suspended in 50 cc. alanine solution.
3. 10.0 grams tissue suspended in 50 cc. phosphate solution.
4. 10.0 grams tissue suspended in 50 cc. phosphate solution.

##### *Leucocytes.*

Approximately equal portions of leucocytes were mixed with

1. 50 cc. alanine solution.
2. 50 cc. alanine solution.
3. 50 cc. phosphate solution.
4. 50 cc. phosphate solution.

In the aseptic kidney experiment only two suspensions were prepared; each minced kidney was suspended in 50 cc. alanine

<sup>10</sup> Levene and Meyer: this *Journal*, xi, p. 365, 1912.

solution. In each instance, suspensions 2 and 4 were coagulated immediately after mixing; 1 and 3 were set aside at 37° for from one to three weeks.

*Methods of analysis.* The contents of each flask were brought to a boil, coagulated and then poured into an excess of absolute alcohol to which was added sufficient 5 per cent alcoholic zinc chloride solution to completely precipitate the protein. This was filtered and the precipitate washed and extracted with hot water, and the filtrate and washing distilled in vacuum to completely remove the alcohol, which was only accomplished by repeating the distillation several times after addition of water. The residue was taken up in water and made up to a definite volume, usually twice that of the original, 50 cc. made up to 100 cc. 10 cc. of this solution were used for a total nitrogen determination, by the Kjeldahl method, 25 cc. for an ammonia determination, and, after removal of the ammonia, for an amino nitrogen determination by the Van Slyke method, as described in detail in a previous communication from this laboratory.

*Bacteriological control.* Aerobic and anaerobic cultures were made of the aseptic mixtures after incubation and smears made of the mixtures with toluene. There was no bacterial contamination. The bacterial examination was made by Dr. H. L. Amoss, and we take this occasion to express our appreciation of his assistance.

#### *Analytical results.*

The amino nitrogen determinations are here reported in detail. The results of the total nitrogen and ammonia nitrogen determinations are included in the appended tables.

#### *Experiment I. Kidney tissue.*

	ORIGINAL SOLUTION USED	GAS VOLUME (CORR.)	TEMPER- ATURE	PRES- SURE	TIME	N	GRAMS PER 100 CC.
	cc.	cc.	°C.	mm.	min.	mgm.	
Alanine solution:							
Before.....	5	26.20	20	751	4	14.74	0.295
After three weeks.....	5	29.20	24	758	4	16.25	0.325
Phosphate solution:							
Before.....	5	1.30	24	760	4	0.78	0.015
After three weeks.....	5	3.30	24	758	4	2.00	0.040

*Experiment II. Kidney tissue.*

	ORIGINAL SOLUTION USED	GAS VOLUME (CORR.)	TEMPER- ATURE	PRESSURE	TIME	N	GRAMS PER 100 CC.
	cc.	cc.	°C.	mm.	min.	mgm.	
Alanine solution:							
Before.....	5	28.00	18	760	4	16.10	0.322
After one week.....	5	30.30	20	758	4	17.30	0.346
Phosphate solution:							
Before.....	5	0.70	18	766	4	0.40	0.008
After one week.....	5	2.80	20	758	4	1.40	0.028

*Experiment III. Liver tissue.*

Alanine solution:							
Before.....	5	27.90	24	760	4	15.60	0.310
After three weeks.....	5	32.20	24	758	4	18.10	0.362
Phosphate solution:							
Before.....	5	2.20	24	760	4	1.28	0.026
After three weeks.....	5	6.20	24	758	4	3.44	0.068

*Experiment IV. Liver tissue.*

Alanine solution:							
Before.....	5	27.80	18	760	4	16.10	0.322
After one week.....	5	29.10	20	758	4	16.50	0.330
Phosphate solution:							
Before.....	5	1.00	18	766	4	0.58	0.012
After one week.....	5	3.30	20	758	4	1.87	0.037

*Experiment V. Leucocytes.*

Alanine solution:							
Before.....	2.5	14.75	22	760	4	8.31	0.332
After three weeks.....	2.5	17.60	20	758	4	19.90	0.398
Phosphate solution:							
Before.....	2.5	0.20	22	760	4	0.113	0.004
After three weeks.....	2.5	3.20	20	758	4	1.81	0.073

*Experiment VI. Aseptic kidney.*

Alanine solution:							
Before.....	2	11.70	20	760	4	6.65	0.332
After three days.....	2	11.70	20	758	4	6.64	0.332

*Experiments with kidney tissue and alanine.  
With toluene.*

	AMMONIA N PER CENT			AMINO N PER CENT			TOTAL N PER CENT		
	Before	After	Diff.	Before	After	Diff.	Before	After	Diff.
I. Three weeks' incubation:									
Tissue and alanine.....	0.003	0.008	0.005	0.295	0.325	0.030	0.350	0.400	0.050
Tissue and phosphate solution.....	0.003	0.006	0.003	0.015	0.040	0.025	0.056	0.100	0.044
II. One-week incubation:									
Tissue and alanine.....	0.004	0.006	0.002	0.322	0.346	0.024	0.336	0.350	0.014
Tissue and phosphate solution.....	0.006	0.010	0.004	0.008	0.028	0.020	0.008	0.0137	0.005

*Experiments with liver tissue and alanine.*

III. Three weeks' incubation:									
Tissue and alanine.....	0.003	0.012	0.008	0.310	0.362	0.052	0.346	0.455	0.109
Tissue and phosphate solution.....	0.005	0.011	0.006	0.026	0.068	0.042	0.045	0.178	0.133
IV. One-week incubation:									
Tissue and alanine.....	0.006	0.010	0.004	0.322	0.330	0.008	0.370	0.434	0.064
Tissue and phosphate solution.....	0.006	0.013	0.007	0.012	0.037	0.015	0.053	0.128	0.075

*Experiments with leucocytes.  
Without antiseptics*

V. Three weeks' incubation:									
Leucocytes and alanine.....	0	0.014	0.014	0.332	0.398	0.066	0.384	0.520	0.136
Leucocytes and phosphate solution.....	0	0.007	0.007	0.004	0.073	0.067	0.056	0.192	0.136

## NOTE ON A CASE OF PENTOSURIA.

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(Received for publication, July 10, 1913.)

The presence of pentose in urine was first demonstrated by Salkowski<sup>1</sup> in 1892. Later Neuberg<sup>2</sup> undertook an investigation into the nature of the pentose. He came to the conclusion that the pentose was *dl*-arabinose. Numerous observers corroborated Neuberg's statement, inasmuch as they found that urine showing the presence of pentose, as a rule, was optically inactive. Only a few instances are recorded in which the urine containing pentose showed optical activity. In the few cases recorded by Luzzatto,<sup>3</sup> Blumenthal,<sup>4</sup> and by Schüler,<sup>5</sup> the urines were dextro-rotatory. Blumenthal and Schüler have taken for granted that the sugar present in the urine was arabinose and hence regarded the pentose observed by them as *l*-arabinose. Luzzatto prepared the phenyl osazone of the pentose present in the urine of his patient and observed a rotation of  $+1.04^{\circ}$  in a concentration which required according to the theory a rotation of  $+1.10^{\circ}$ . On the basis of this he came to the conclusion that the sugar was *l*-arabinose.

Very recently Elliott and Raper<sup>6</sup> reported on a case of pentosuria in which the sugar failed to form derivatives with diphenyl hydrazine, nor did they succeed in obtaining any other evidence that the sugar was arabinose, and in fact suggested the possibility that the sugar was ribose.

A few years ago a urine containing a pentose came to our observation. A phenyl osazone prepared from the sugar showed

<sup>1</sup> Salkowski and Jastrowitz: *Centralbl. f. d. med. Wiss.*, xxx, p. 337, 1892.

<sup>2</sup> Neuberg: *Ber. d. deutsch. chem. Gesellsch.*, xxxiii, p. 2243, 1900.

<sup>3</sup> Luzzatto: *Hofmeister's Beiträge*, vi, p. 87, 1905.

<sup>4</sup> Blumenthal: *Realenzyklopädie*, xxxii, p. 388, 1908.

<sup>5</sup> Schüler: *Berl. klin. Wochenschr.*, xlvii, p. 1322, 1910.

<sup>6</sup> Elliott and Raper: *this Journal*, xi, p. 211, 1912.

dextro-rotation of  $+0.86^{\circ}$  in a concentration and in a solution prepared according to the directions of Neuberg. Under such conditions *l*-arabinose shows a rotation of  $+1.10^{\circ}$ , and *l*-xylose a rotation of  $+0.15^{\circ}$ . Hence it was evident that the pentose present in the urine under our observation belonged to the arabinose group. In fact, very frequently in course of the work on nucleic acid phenyl osazone of *d*-ribose was obtained which in the same concentration and in the same solution as used in the present experiment showed an identical rotation of  $-0.86^{\circ}$ . An attempt was therefore made to establish the nature of the pentose. Great difficulties, however, were encountered in the attempt to obtain the sugar in a higher concentration. The urine contained only 0.25 per cent of the pentose (calculated on the basis of glucose). At first an attempt was made to concentrate the urine at a pressure of about 18 mm. and a temperature of about  $40^{\circ}$ . However, when the urine was concentrated to a thick syrup, it gave only a slight test with orcin and possessed only insignificant reducing power for Fehling's solution. Evidently the greater part of the pentose was either destroyed or had undergone some condensation so that the usual derivatives of it were no longer obtainable.

It is worthy of note that Luzzatto had already called attention to the fact that the concentrating of the urine of his patient led to a considerable loss of the pentose. It was necessary to find a way by which the sugar could be obtained in greater concentration than present in the urine before any more detailed investigation into the nature of the pentose could be undertaken. It was found that the addition of weak acids or of barium carbonate to the urine to some extent improved the condition so that the pentose could be obtained in concentration of 1 to 2 per cent. But, even then, the concentrated urine showed the evidence of considerable decomposition of the pentose.

An attempt was then made, first to remove the urea of the urine and then to obtain the pentose by means of a solution of lead acetate and of barium hydrate. The lead precipitate of the pentose was then decomposed in the usual way and the aqueous solution containing the sugar concentrated under diminished pressure and at a low temperature. Solutions obtained in that manner contained very little nitrogen but at the end of concentration

again showed a considerable destruction of the pentose. Also this method, therefore, was abandoned. Finally it was concluded to dry the lead precipitate of the sugar and then to extract it with alcohol containing just sufficient hydrochloric acid to remove the lead. In this manner an alcoholic solution of the pentose was obtained. It was hoped that this alcoholic solution of the pentose could be used directly for the preparation of the various hydrazones. However, in no instance was it possible to obtain a diphenyl hydrazone characteristic for arabinose. Even in solutions which contained the pentose in a fairly high concentration an insoluble hydrazone could not be obtained. On the other hand, the addition of arabinose either directly to the urine, or to the concentrated urine, or to the solutions obtained on decomposition of the lead derivatives always led to the formation of the hydrazone. The failure to obtain the diphenyl hydrazone naturally can be interpreted in two ways: first, that the urine contained some substances which prevented the hydrazone from crystallizing; and second, that the pentose was not arabinose, but either ribose or keto-arabinose. It is worthy of note that the residue obtained on evaporation of the alcoholic extract from the lead compound of the sugar contained a large proportion of organic on-nitrogenous material which was not the pentose. It is undoubtedly due to the presence of these substances that a crystalline hydrazone of the pentose was not obtainable. It was also impossible to obtain in crystalline form an oxidation product of the pentose.

In order to obtain some information into the nature of the pentose the optical rotation of the sugar solution was compared with its reducing power for Fehling's solution. In this manner it was found that the specific rotation of the sugar was  $+17.5^\circ$ . The specific rotation of *l*-arabinose is  $+104.4^\circ$  and that of ribose  $19.3^\circ$ . On the basis of this it seems more probable that the pentose present in the urine under our observation is not *l*-arabinose but *l*-ribose. We fully realize, however, that the exact nature of the sugar cannot be made certain before it is obtained in purity that will permit the obtaining of some well-defined derivatives.

A report is made at this stage for the reasons, that on the one hand the material is not accessible for further work, and second

that it suggests the possibility that there may exist more than one form of pentosuria.

#### EXPERIMENTAL.

##### Osazone.

400 cc. pentose urine were heated on the water bath with 5 grams of phenyl hydrazine. The osazone began to separate almost at once and after one hour the crystals were filtered off and recrystallized from water containing pyridine. It melted at 163°C.

0.1264 gram substance gave 0.0712 gram  $H_2O$  and 0.2872 gram  $CO_2$ .

	Calculated for $C_{12}H_{12}O_6N_4$ :	Found:
C.....	62.19	61.98
H.....	6.10	6.24

0.1002 gram osazone in 5 cc. pyridine alcohol rotated in a 0.5 dm. tube with D-light + 0.43°.

5 liters of urine were precipitated with mercuric acetate and sodium hydrate. The mercury was removed from the filtrate with hydrogen sulphide and a small amount of lead acetate solution added to remove the excess of the latter. The filtrate was then treated alternately with a solution of basic lead acetate and barium hydrate until no further precipitate was produced. The lead and barium precipitate was washed with water and decomposed by shaking with 5 per cent sulphuric acid. The excess of the sulphuric acid was removed with barium carbonate and the amount of sugar calculated as glucose found by titration to be 3 grams. The solution could not be concentrated without destruction of the sugar.

2 cc. of solution corresponded to 13.5 cc. ammonium sulphocyanate (1 cc.  $\frac{N}{10}$  ammonium sulphocyanate = 377 mgm. ribose).<sup>7</sup> Hence, 100 cc. contained 2.54 grams calculated from the absolute reducing power of ribose. It rotated in a 2 dm. tube with D-light + 0.91°.

$$[\alpha]_D^{20} = +17.5^\circ$$

1 PER CENT RIBOSE SOLUTION	FEHLING'S SOLUTION	$\frac{N}{10}$ SULPHOCYANATE	FOR 1 CC.
cc.	cc.	cc.	
2	14	4.70	2.35
3	21	8.30	2.76
4	28	10.40	2.60
4	28	10.60	2.65
5	35	13.30	2.66
7	49	18.50	2.60

<sup>7</sup> The exact data regarding the reducing power of ribose for Fehling's solution are as yet lacking. The following experiments were made prin-



*Concentration of the pentose.*

By extracting the dried precipitate from the lead and barium with warm alcoholic hydrochloric acid a solution of the pentose was obtained, which after removal of the hydrochloric acid with an excess of lead carbonate could be concentrated to a syrup which is free from nitrogen. However, the total weight of the residue exceeded enormously the calculated weight of sugar in it.

*Attempts to prepare hydrazones of the pentose.*

20 cc. of a solution containing 0.4 gram sugar were treated with 0.5 gram parabromophenyl hydrazine and the solution concentrated in vacuum at room temperature in a desiccator. A syrup was left which did not crystallize.

The experiment was performed with benzyl phenyl hydrazine with addition of an equal volume of alcohol and boiling for one hour on the water bath. Upon concentrating in vacuum only a syrup was obtained which did not crystallize.

The same experiment with diphenyl hydrazine also led to a negative result. The last two experiments were repeated. The addition of 0.4 gram of arabinose yielded the respective hydrazones.

5.6 grams sugar in 750 cc. of water were treated with 3.9 grams (1 mol.) phenyl hydrazine and the solution evaporated at low temperature in vacuum. Instead of a hydrazone the above described osazone was obtained.

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cipally to obtain the factor required for the present work. More detailed and numerous determinations are still needed. The reducing power of ribose was first determined approximately by varying the amount of Fehling's solution required to reduce a given amount of 1 per cent solution, and the following determinations carried out by Volhard's method, using the required volume of the same concentration of Fehling's solution.

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## A NEW METHOD FOR THE DETERMINATION OF UREA IN BLOOD.

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(Received for publication, July 14, 1913.)

In a recent number of this *Journal*,<sup>1</sup> a new method for the estimation of urea in urine was described. This method consists in the conversion of the urea into ammonium carbonate by means of the urease of the soy bean, and an estimation of the alkalinity before and after the conversion by means of standard acid and methyl orange. The procedure is not directly applicable to the estimation of urea in blood serum owing to the large quantity of proteins and small amount of urea present. However, by a conversion of the urea into ammonium carbonate with the use of the enzyme, and a subsequent removal of the ammonia by means of an air current as suggested by Folin,<sup>2</sup> satisfactory results can be obtained without a preliminary removal of the proteins, as is necessary in other methods for the determination of urea in blood.

*Procedure.* The blood is drawn in the usual manner and allowed to stand on ice until clotting is complete. As shown below the urea content of the serum does not change after standing even for three or four days; the blood can, therefore, be kept on ice over night, if desired.

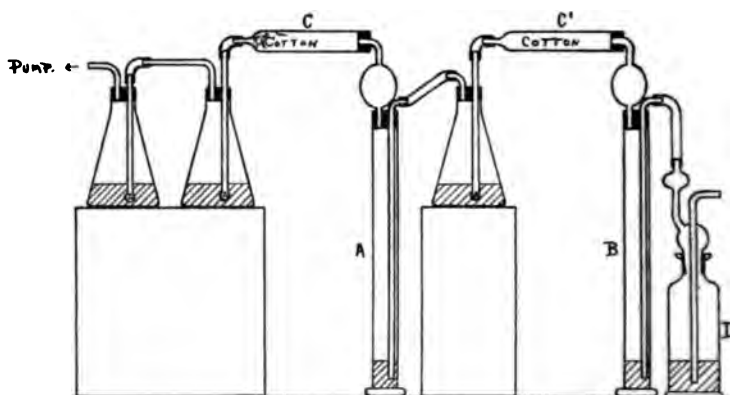
Two equal portions of the serum are measured into ordinary test tubes, 1 cc. of the soy bean extract<sup>3</sup> added to one tube, and

<sup>1</sup> This *Journal*, xiv, p. 283, 1913.

<sup>2</sup> *Zeitschr. f. physiol. Chem.*, xxxvii, p. 161, 1902-03.

<sup>3</sup> The preparation of the soy bean extract is described in the previous article. Ten grams of finely ground soy beans are treated with 100 cc. of water and allowed to stand with occasional agitation for one hour. 10 cc. of  $\frac{7}{10}$  hydrochloric acid are added and the mixture allowed to stand about fifteen minutes longer. It is now filtered and preserved with toluene. Such a solution is perfectly satisfactory for use at least five or six days after its preparation.

about 0.5–1.0 cc. of toluene to each. If sufficient serum is available, 10-cc. portions should be used; however, perfectly satisfactory results can be obtained by using 5-cc. or even 3-cc. portions of the serum. The tubes are tightly stoppered, and allowed to remain at room temperature until the conversion of the urea into ammonium carbonate is complete. Generally, they are allowed to stand over night, although four to five hours is usually amply sufficient for the completion of the reaction. The contents of the tube containing the serum and extract are transferred to cylinder A, and washed in with a very small amount of water (not more



than 5 cc.). Two grams of sodium chloride, an equal volume of alcohol and a layer of kerosene oil are added to the cylinder. The contents of the other tube are transferred to cylinder B, and treated in exactly the same manner. 25 cc. of  $\frac{N}{5}$  hydrochloric acid and about 25 cc. of water are placed in each of the 200 cc. Erlenmeyer flasks used for the absorption of the ammonia. The different parts of the apparatus are now connected and about 0.5 gram of sodium carbonate added to each cylinder. A rapid air current is passed through the apparatus until all the ammonia has been removed from the cylinders. With a good suction pump, one hour suffices. The excess of acid in the absorption flasks is titrated with  $\frac{N}{5}$  sodium hydroxide and alizarin sodium sulphonate. The amount of acid neutralized in the flask attached to cylinder

*B* corresponds, of course, to the ammonia<sup>4</sup> present in the serum, while the amount used in the other two flasks represents the urea plus the ammonia. The difference corresponds to the urea in terms of  $\frac{N}{30}$  hydrochloric acid, and multiplied by 0.0006 gives the urea in grams present in the amount of serum taken for the determination.

*Details in connection with the apparatus and determination.* 1. On account of the large quantity of protein in serum, it was found advisable to use both alcohol and kerosene to prevent foaming.<sup>5</sup>

2. The tubes *C* and *C'* are ordinary calcium chloride drying tubes packed loosely with cotton. These in conjunction with the bulbs prevent any splashing or mechanical transmission of the alkali into the absorption flasks. While the bulbs are probably not absolutely necessary, they are convenient in keeping the cotton filters dry.

3. For the better absorption of the ammonia, the tubes in the Erlenmeyer flasks are closed at one end, and pierced with six or seven small holes, as suggested by Folin.<sup>6</sup> Even with this device one absorption flask is not always sufficient to completely absorb the ammonia. Two flasks are always used for safety in connection with the urea determination; however, since from the serum alone only a very small amount of ammonia (corresponding to 0.10–0.70 cc. of  $\frac{N}{30}$  HCl) is ordinarily obtained, one absorption flask is here sufficient.

4. A layer of toluene is placed on the liquid in the absorption flasks, for, due probably to the alcohol carried over by the air current, considerable foaming sometimes occurs. If not prevented, this results in a loss of a portion of the contents of the flask.

5. The bottle *D* contains dilute sulphuric acid to free the air from any traces of ammonia before passing it through the apparatus.

<sup>4</sup> We can, however, place no value on this as a determination of the true ammonia content of the blood; for on standing even a few hours the blood develops much more ammonia than the original amount (Folin: *this Journal*, xi, p. 527, 1913).

<sup>5</sup> This has been earlier pointed out by Folin in connection with the use of the air-current method for determining ammonia in blood (*Zeitschr. f. physiol. Chem.*, xxxvii, p. 165, 1902-03).

<sup>6</sup> *This Journal*, xi, p. 493, 1912.

6. No correction is necessary for the ammonia derived from the 1 cc. of soy bean extract used, as the amount obtained from this source is inappreciable.

The following table shows the results obtained with various preparations of the extract.

AGE OF EXTRACT	AMOUNT TAKEN	$\frac{N}{50}$ HCl REQUIRED	$\frac{N}{50}$ HCl CAL. FOR 1 CC.
	cc.	cc.	cc.
fresh	2	0.04	0.02
fresh	10	0.20	0.02
18 hrs.	5	0.28	0.06
3 days	10	0.33	0.03
5 days	4	0.13	0.03

*Determination of pure urea solutions.* The following table presents the results obtained by the application of this method to very small amounts of urea, and also to mixtures of urea and ammonium sulphate.

GRAMS UREA TAKEN	GRAMS UREA FOUND	GRAMS UREA TAKEN	GRAMS UREA FOUND
0.00066	0.00069	0.01100	0.01080
0.00066	0.00072	0.01120	0.01110
0.00110	0.00114	0.01120	0.01193
0.00110	0.00108	0.01263	0.01278
0.00330	0.00310	0.01263	0.01243
0.00506	0.00507	0.02526	0.02526
0.00550	0.00550	0.02526	0.02523

GRAMS UREA TAKEN	GRAMS $(\text{NH}_4)_2\text{SO}_4$ TAKEN	GRAMS UREA FOUND	GRAMS $(\text{NH}_4)_2\text{SO}_4$ FOUND
0.00132	0.00079	0.00138	0.00084
0.00220	0.00132	0.00217	0.00134

*Determination of urea added to blood.* Varying small amounts of urea were added to blood serum and the amount of urea estimated before and after the addition. Such a procedure demonstrates that the method is capable of yielding all the urea present in the serum; however, it does not prove that other substances may not be undergoing decomposition at the same time. Tacheuchi

found that the urease of the soy bean was not capable of yielding any ammonia from guanidine, arginine, benzamide, allantoin, leucine, alanine, tyrosine, creatine, histidine, guanine, glycocoll, uric acid or hippuric acid.<sup>7</sup> Hence it appears very probable that no other constituents of the blood (or urine) contribute ammonia to the final result when urea is determined by this method.

SOURCE OF BLOOD	UREA: GRAMS IN 5 CC. SERUM	UREA ADDED TO 5 CC. SERUM	UREA FOUND	DIFFERENCE: ADDITIONAL UREA
Pig.....	0.00066	0.00510	0.00566	0.00500
	0.00071	0.00517	0.00585	0.00514
	0.00071	0.00517	0.00589	0.00518
	0.00069	0.00517	0.00573	0.00504
	0.00069	0.00517	0.00582	0.00513
	0.00150	0.01000	0.01092	0.00942
	0.00072	0.01000	0.01074	0.01002
Dog.....	0.00157	0.00505	0.00654	0.00497
	0.00157	0.01263	0.01395	0.01238
	0.00138	0.01263	0.01318	0.01180
	0.00110	0.00505	0.00621	0.00511
	0.00070	0.00208	0.00267	0.00197

Duplicate determinations by this method agree quite closely. On a sample of human blood was obtained 0.477 and 0.483 gram per liter; on a sample of pig's blood, 0.144, 0.144, 0.141, 0.147 gram per liter; and on a sample of dog's blood, 0.276, 0.267, 0.270 gram per liter. A considerable number of determinations have been made by this method on human blood under pathological conditions and will be reported elsewhere.

*Stability of urea in serum.* Since in the method outlined above, the serum is allowed to stand over night, it is of great importance to determine whether the urea content of the serum is diminished by this procedure. The figures cited below prove that serum allowed to remain on ice for even four days does not appreciably change in its urea content. The serum was allowed to remain in an ice chest, portions pipetted off at various intervals and the urea estimated. The figures represent grams of urea per liter of serum.

<sup>7</sup> *Journal of the College of Agriculture, Tokyo*, i, p. 1, 1909.

*Pig's serum:* April 22, 0.144; April 23, 0.141; April 24, 0.138; April 26, 0.147.

*Dog's serum:* (a) April 25, 0.174; April 28, 0.177 (b) May 10, 0.279; May 12, 0.276.

Also, the stability of urea in serum is shown by the fact that if urea is added to serum and its value determined immediately, and then the serum allowed to stand over night at room temperature and the urea determined, practically the same figures are obtained. In the first instance, in the following table, the urea was determined at once by adding 1 cc. of the extract and allowing the serum to stand for four hours; in the other two cases the serum was allowed to remain over night before estimating the urea.

GRAMS UREA IN 5 CC. SERUM	GRAMS UREA ADDED TO 5 CC.	GRAMS UREA FOUND	DIFFERENCE
0.000705	0.00517	0.00571	0.00501
0.000705	0.00517	0.00585	0.00515
0.000705	0.00517	0.00588	0.00518

However the amount of acid required for the blank increases, that is, the ammonia present in the serum is increased. The increase in ammonia does not come from the urea, but from the proteins or some other source.

*Comparison of this method with Folin's method.* A few comparative figures of the results obtained by this method with those obtained by Folin's new method<sup>8</sup> for determining urea in blood, are cited below. Dog's blood serum was used in each instance. Since the blood was allowed to clot, and stand for four or five hours, its ammonia content was, of course, increased. In Folin's method the blood is transferred immediately to methyl alcohol as soon as it is drawn, consequently no correction for the ammonia present is necessary. Here, however, we would have a correction for the ammonia, but since the ammonia determinations were not made at the same time as the serum was transferred to methyl alcohol, we have preferred to give the uncorrected results. This explains in some measure the higher results obtained by

<sup>8</sup> This *Journal*, xi, p. 527, 1912.



Folin's method. All results are calculated in grams of urea per liter of serum.

Urease method.....	0.372	0.360	0.180	0.324	0.278	0.291	0.288
Folin's method.....	0.408	0.396	0.234	0.321	0.310	0.338	0.300

*Application of the method to body fluids and tissues.* An application of the method described in this paper to the more exact determination of urea in urine than is afforded by the rapid clinical method previously described is shown in a following paper. The method should lend itself readily to the estimation of urea in other body fluids and tissues. In fluids, the urea can generally be determined without preliminary treatment; while tissues must be first extracted with alcohol, the alcohol extract evaporated, and the residue dissolved in water before applying the method. The question of inhibitory substances must, of course, be considered in this connection. If a liquid contains acids or mercuric chloride in appreciable amount, the presence of these will completely arrest the action of the enzyme. The following procedure is useful in testing a fluid for inhibitory substances. A quantity of the fluid, equal in amount to that taken for the determination is measured into a test tube, a couple of drops of phenolphthalein solution added, and a few crystals of pure urea. 1 to 2 cc. of the soy bean extract are added and provided no inhibitory substances are present, the red color of the indicator should appear in about five to ten minutes. Armstrong has recently<sup>9</sup> studied the action of certain substances on urease. Of the substances studied, quinone, quinol and formaldehyde had an extreme inhibitory effect, while amino-acids and other weak acids in small amount accelerated the action slightly.

A few results showing the application of the method to the determination of urea in sweat are cited below.

UREA FOUND IN 5 CC. SWEAT	UREA ADDED TO 5 CC. SWEAT	UREA FOUND	DIFFERENCE
0.0140	0.00416	0.01812	0.00412
0.00825	0.00552	0.01359	0.00534

<sup>9</sup> Armstrong, Benjamin and Horton: *Proc. Roy. Soc., B*, lxxxvi, p. 328, 1913.

A more extensive investigation of the action of acids and alkalis on this enzyme, as well as its behavior in the presence of various substances, will be investigated to determine the value and limitations of the method in estimating urea in miscellaneous fluids.

## THE DETERMINATION OF UREA IN URINE.

(SECOND COMMUNICATION.)

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(Received for publication, July 14, 1913.)

The method outlined in the foregoing paper for the estimation of urea in blood is perfectly reliable for larger quantities of urea (up to 25 mgms. or perhaps more). Its adaptation to the determination of urea in urine using only a small amount of the urine is shown in this paper. The method to be described is slightly more accurate than the rapid clinical method proposed earlier.<sup>1</sup> The reason for this is twofold; the rapid titration method gives results slightly lower than the theoretical on account of the presence of carbon dioxide during the titration, and titrations performed in a mixture such as urine are not as accurate as in the case of the neutralization of a pure solution of an acid by an alkali.

Two 1-cc. portions of the urine are measured into test tubes by means of an Ostwald pipette.<sup>2</sup> The urine is now diluted to about 10 cc., 1 cc. of the soy bean extract added to one tube and from 0.5–1 cc. of toluene to each. The tubes are allowed to remain at room temperature over night. With the following exceptions, the remainder of the procedure is exactly similar to that described in the foregoing article on the determination of the urea in blood. No alcohol need be added to the contents of the cylinders, for oil alone suffices to prevent excessive frothing. The first absorption flask attached to the cylinder containing the urine and the ferment extract is charged with 50 cc. of  $\frac{N}{50}$  hydrochloric acid instead of 25 cc. In the case of pathological urines

<sup>1</sup> This *Journal*, xiv, p. 483, 1913.

<sup>2</sup> These pipettes are the same as those used by Folin (*this Journal*, xi, p. 493, 1912) and can be obtained from Eimer and Amend. However, should 5 cc. of the urine be available, it is equally convenient to dilute this to 50 cc. and use two 10-cc. portions for the determination.

containing large amounts of ammonia two absorption flasks may be necessary for each cylinder.<sup>3</sup>

Instead of the foregoing procedure in which the urea and ammonia are determined in one operation, the ammonia can be obtained by the air-current method in the usual manner using larger amounts of urine. Then, only one portion of the apparatus is necessary for the urea estimation.

A comparison of the results obtained by this method, the rapid clinical method previously described, and the new phosphoric acid method<sup>4</sup> proposed by Folin is given in the table below. Folin<sup>5</sup> has recently compared the phosphoric acid method with his magnesium chloride method and Benedict<sup>6</sup> method, and found that the three methods yield practically identical results. In using the phosphoric acid method, two absorption flasks were found necessary instead of one. The results are expressed in grams of urea per liter of urine.

RAPID METHOD	NEW METHOD	FOLIN'S PHOSPHORIC ACID METHOD
19.50	19.80	19.86
11.80	12.00	11.60
7.98	8.04	7.95
15.12	15.12	15.18
25.46	25.08	25.08
11.10	11.37	11.10
19.62	19.80	19.68
	15.09	15.00
19.65	20.19	20.25
21.42	22.02	21.66
9.06	9.15	9.12
19.20	19.38	19.00
19.50	20.22	20.28

Hence, it appears from the above table that the rapid method gives results which are perfectly reliable, while the results obtained by the method here described and Folin's phosphoric acid method are practically the same.

<sup>3</sup> Of course the special ammonia-absorption tubes devised by Folin might be used and hence only one absorption cylinder would be necessary in each case. However, with the very dilute solutions used for titration, it appeared better to keep the volume as small as possible.

<sup>4</sup> This *Journal*, xi, p. 512, 1912.

<sup>5</sup> *Ibid.*, xi, p. 511, 1912.

<sup>6</sup> *Ibid.*, viii, p. 405, 1910.

**BLOOD GLYCOLYSIS: ITS EXTENT AND SIGNIFICANCE  
IN CARBOHYDRATE METABOLISM. THE SUPPOSED  
EXISTENCE OF "SUCRE VIRTUEL" IN FRESHLY  
DRAWN BLOOD.**

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(Received for publication, July 24, 1913.)

After a lapse of several years during which it was largely neglected, the subject of glycolysis has recently received considerable attention and with important and far-reaching results.<sup>1</sup> These researches have dealt with both tissue and blood glycolysis. In connection with the former although much has been added to our knowledge of the rate of sugar consumption by the surviving heart it has, so far, been impossible to demonstrate that true glycolytic power exists in the case of isolated animal tissues or juices.<sup>2</sup>

The greater part of the work within the past year or so has been concerned with blood glycolysis and it may be of interest to review some of the results that have been obtained. In the first place it seems clear that the activity of the glycolytic process varies extremely in the blood of different classes of animals. Thus A. Loeb<sup>3</sup> has found that glycolysis is most marked in the blood of the dog, being also considerable in that of man and sheep; on the other hand it has been found almost absent in the blood of the ox and pig.<sup>4</sup>

<sup>1</sup> For full literature concerning the older researches consult Oppenheimer: *Die Fermente*, 2er Aufl. (Vogel, Leipzig, 1910); for more recent work, von Fürth: *Probleme der physiologischen und pathologischen Chemie*, Bd. ii (Vogel, Leipzig, 1913).

<sup>2</sup> Levene and Meyer: *This Journal*, xi, pp. 347-353, 1912.

<sup>3</sup> Loeb, A.: *Biochemische Zeitschr.*, xlix, p. 413, 1913.

<sup>4</sup> Cf. also Melvin, G. S.: *Biochemical Journ.*, vi, p. 422, 1912. Melvin could detect no glycolysis either in ox- or sheep-blood.

It has been pointed out by this observer that the bloods which exhibit feeble glycolytic power are those in which normally the red corpuscles contain only a small percentage of dextrose, it having been previously established by Masing<sup>5</sup> that the distribution of dextrose between corpuscles and plasma varies very considerably in the blood of different classes of animals. After the addition of dextrose to blood, the corpuscles of which normally contain little dextrose (and which therefore possess little glycolytic power) no evidence of penetration of the dextrose into the corpuscles could be obtained by Masing. Taking all the facts together, Loeb has formulated the hypothesis that the glycolytic process resides within the red corpuscles, through the envelope of which the sugar must therefore pass before it can be destroyed. Two factors might therefore influence the rate of glycolysis in whole blood: the diffusibility of the corpuscular envelope towards sugar and the glycolytic power of the corpuscular contents. By observations on the relative disappearance of dextrose from serum and corpuscles respectively, Loeb has furnished very strong evidence in favor of his hypothesis, having found that the amount of sugar in the corpuscles shows only a slight decrease, but that in the serum the decrease is marked, in those bloods (dog) in which the envelope is very permeable towards this substance. While not denying that erythrocytes may possess glycolytic powers, Levene and Meyer have shown that leucocytes are also endowed with this property and Rona and Arnheim<sup>6</sup> have found that both leucocytes and erythrocytes of "oxalate" blood of the dog and cat can destroy dextrose in the presence of phosphates. It was impossible in these observations to determine in which variety of corpuscle this power was the more marked.

Among other important recently established facts regarding blood glycolysis may be cited the following:

1. Serum has no glycolytic power (Rona and Döblin,<sup>7</sup> von Noörden, Jr.<sup>8</sup>).

2. The glycolytic power of the blood of different individuals of the same species may vary considerably. For example, in the

<sup>5</sup> Masing: *Pflüger's Archiv für die gesamte Physiologie*, cxlix, p. 227, 1912.

<sup>6</sup> Rona and Arnheim: *Biochem. Zeitschr.*, xlviii, p. 35, 1913.

<sup>7</sup> Rona and Döblin: *ibid.*, xxxii, p. 489, 1911.

<sup>8</sup> von Noörden, Jr.: *ibid.*, xlv, p. 94, 1912.

case of the blood of the dog the following values, expressing the disappearance of dextrose as a percentage of the amount of dextrose originally present (percentile glycolysis) have been found.

After 5 hours at 37°C., between 25 and 26.

After 6 hours at 37°C., between 20.6 and 41.6 (Rona and Arnheim).

After 1½ hours at 40°C., between 48.5 and 62.7 (A. Loeb).

After 2 hours at 37°C. (2 per cent oxalate), between 15 and 18.6.

After 6 hours at 37°C., between 59.7 and 88.4 (Edelmann).<sup>9</sup>

After 90 minutes at 40°C., between 33 and 58.

After 2 hours at 40°C., between 30 and 32 (Kondo).<sup>10</sup>

3. Although the glycolytic process has been said to be depressed by the addition of fluoride to the blood, it is claimed by certain authors that oxalates in sufficient amount to prevent clotting do not have this effect. Rona and Arnheim<sup>11</sup> state that 0.1 per cent oxalate causes no depression. Edelmann employed blood containing 0.2 per cent oxalate, but, as a general rule, the glycolysis in his observations was slower than that observed by other workers.

4. Dilution of blood with water destroys the glycolytic power, which is not the case when the dilution is made with Ringer's solution or with a phosphate mixture. Besides the H ion concentration, the phosphate and carbonate ions seem to be important for the process. Mere laking of the blood, as by means of saponin, does not destroy the glycolytic power.<sup>12</sup>

5. Although oxygen favors glycolysis to a certain extent, it can nevertheless proceed anaerobically (Rona and Döblin). The glycolytic power disappears after the blood has stood for some time outside the body.

6. The addition of dextrose to blood does not increase the absolute amount of glycolysis occurring in a given time; it therefore lowers the percentile glycolysis (Rona and Döblin).<sup>13</sup>

In the present research several of the conclusions mentioned in the above review have been put to the test and observations have been made with the object of demonstrating whether the process of blood glycolysis plays any rôle in the consumption of carbohydrate in the intact animal.

<sup>9</sup> Edelmann: *Biochem. Zeitschr.*, xl, p. 314, 1912.

<sup>10</sup> Kondo: *ibid.*, xlv, p. 88, 1912.

<sup>11</sup> Rona and Arnheim: *loc. cit.*

<sup>12</sup> Edelmann: *loc. cit.*

<sup>13</sup> Rona and Döblin: *loc. cit.*

## EXPERIMENTAL.

*Methods.* The blood from the femoral vein or artery of etherized dogs was collected in sterile flasks, under strict aseptic precautions, and the animal was, as a rule, allowed to recover from the operation. In most of the experiments the blood was defibrinated by means of glass beads and quantities of 15 cc. each were transferred to small sterile Erlenmeyer flasks which, after being closed by sterile cotton plugs and tin foil, were suitably fixed to a holder which was kept in motion by means of a motor. The holder was placed in a water bath at 40°C. After varying periods of time the flasks were removed and the sugar was determined in the incubated blood by Bertrand's method, the proteins having been first of all removed by means of colloidal iron. The sugar was similarly determined in a control sample of blood before incubation. Although in the few hours during which the blood was incubated in our experiments, there could have been little danger of bacterial destruction of sugar, yet in all the earlier observations careful bacteriological examinations were made, but with negative results. For those examinations we are indebted to Mr. W. W. Donaldson.

*Glycolysis in oxalate and in defibrinated blood.*

It was found that the method employed for preserving the blood in a fluid condition has an influence on the rate of glycolysis in that oxalate, beyond a concentration of one per thousand, distinctly retards it. This is shown in the following table in

TABLE I.

NO. OF EXP.	TIME OF INCUBA- TION	OXALATE BLOOD		DEFIBRINATED BLOOD			
		Per cent dextrose in blood at start	Per cent oxalate	Dextrose dis- appeared from 100 gms. blood	Percentile glycolysis	Dextrose dis- appeared from 100 gms. blood	Percentile glycolysis
	hours			grams		grams	
	3	0.126	0.1	0.017			
	5			0.034	27	0.079	66.8
	4½		0.34	0.033	16.6	0.085	63



which are given the amounts of dextrose disappearing from 100 grams of blood and the *percentile glycolysis*<sup>14</sup> for blood that was either received into a sterile solution of potassium oxalate or was defibrinated in a sterile flask by shaking with glass beads.

The following table shows that the depression of glycolytic power is more or less proportional to the concentration of oxalate.

TABLE II.

NO. OF EXP.	TIME OF INCUBATION	PER CENT DEXTROSE IN BLOOD AT START	PER CENT OXALATE	DEXTROSE DISAPPEARED FROM 100 GMS. BLOOD	PERCENTILE GLYCOLYSIS
	<i>hours</i>			<i>grams</i>	
2	2	0.195	0.128	0.011	5.6
	5½			0.092	47.2
2	2	0.200	0.178	0.008	4.8
	5½			0.068	33.8
1	2	0.216	0.4	0.004	
	3½			0.021	9.2

The depressing influence of the oxalate is not dependent on the fact that clotting has been prevented but is due to an influence of the oxalate itself. This was demonstrated by studying the effect produced on glycolysis by adding various amounts of oxalate to defibrinated blood and by comparing the glycolysis in unclotted "hirudin" blood with that occurring in another sample of the same blood after defibrination. Table III gives the results of these experiments.

Although we cannot say from the above experiments whether it would be possible to add a sufficiency of oxalate to prevent clotting without interfering with glycolysis, yet it is evident that with the amount of this substance usually employed for this purpose (viz., 0.1 per cent) considerable interference occurs. If unclotted blood is required, as when the glycolytic powers of corpuscles and plasma are to be separately investigated, hirudin should therefore be employed.

The same influence of oxalate on glycolysis was observed in blood which had been laked by means of saponin. Thus in laked blood containing about 0.5 per cent oxalate only 0.004 gram of dextrose disappeared in three hours, whereas without oxalate

<sup>14</sup> Percentile glycolysis means the decrease of dextrose per 100 grams of blood as a percentage of the amount of dextrose originally present.

0.0405 gram of dextrose disappeared, the original percentage of dextrose in both cases being 0.096.

TABLE III.

NO. OF EXP.	TIME OF INCUBA- TION	PER CENT DEXTROSE IN BLOOD AT START	DEFIBRINATED BLOOD		DEFIBRINATED BLOOD + OXALATE OR HIRUDIN		
			Dextrose disap- peared from 100 gms. blood	Percentile glycolysis	Per cent oxalate or hirudin	Dextrose disap- peared from 100 gms. blood	Percentile glycolysis
	<i>hours</i>		<i>grams</i>			<i>grams</i>	
5	3	0.101	all?	100?	oxalate 0.125	0.042	41.7
7	2½	0.154	0.084	54.2	oxalate indefinite	0.053	34.4
8	½	0.079	0.038	48.2	hirudin 1 mg. to 8 gms.	0.023	29.8
	2½		0.044	55.6		0.056	71.8
	4½		0.064	81.2		0.064	81.2
9	½	0.162	0.021	12.9	hirudin, 1 mg. to 8 gms.	0.021	12.9
	2½		0.077	47.5		0.081	50

*The average rate of glycolysis in defibrinated blood at 40°C.*

Although a great part of the discordance between the results of previous observers as to the intensity of glycolysis is to be explained by differences in the method used for keeping the blood fluid, and in the source of the blood, yet, even in the case of defibrinated dog-blood, glycolysis does not always proceed at the same rate. To study the variations we have compared: (1) the glycolysis occurring in blood collected at different periods from the same dog living meanwhile under practically constant conditions and (2) that occurring in the blood of different dogs. The values given in the following table were found in the blood of four dogs observed at different periods:

TABLE IV.

EXP. NO.	DATE	TIME OF INCUBATION	PER CENT DEXTROSE IN BLOOD AT START	DEXTROSE DISAPPEARED FROM 100 GMS. BLOOD	PERCENTILE GLYCOLYSIS
		<i>hours</i>		<i>grams</i>	
A 3 15	III, 3	5	0.119	0.079	66.8
	III, 27	3	0.134	0.083	
		5½		0.103	76.5
B 2 22	III, 4	2½	0.129	0.059	45.4
	IV, 21	2½	0.106	0.047	44.2
C 5 17	III, 11	3	0.101	all?	
	IV, 7	3	0.104	0.071	
D 8  12	III, 17	2½	0.079	0.044	55.6
		4½		0.064	81.1
	III, 21	3	0.205*	0.086	42.4
		5		0.130	63.4

\* Hyperglycaemia.

It is seen that distinct variations occur when we consider the absolute amounts of dextrose that disappear during each period. More constant values are however obtained when the decrease is calculated as a percentage of the amount of dextrose originally present (percentile glycolysis). Although such a calculation is probably permissible when the percentage of dextrose is at or about the normal, this is not the case when the percentage is excessively high. We shall give evidence for this statement later (see page 508), meanwhile it is important to note that the discrepancies in dog D are probably to be explained in this way.

Turning now to the observations on different animals, Table V contains figures expressing the percentile glycolysis occurring at different periods in cases where the original percentage of dextrose in the blood was approximately normal.

From such results we cannot form any very accurate estimate of the average velocity of the glycolytic process although in general it is probably safe to conclude that approximately one-quarter of the sugar has disappeared in about one and one-half and about one-half of it in about two and one-half hours.

We have not a sufficient number of observations on any one sample of blood, from which it is possible to construct the velocity

curve of the glycolysis but it is perfectly clear, especially from such results as are given in Table V, that the process is much quicker during the first hour or so than later. It probably proceeds very slowly during the later stages: In one experiment (No. 21) for example, blood incubated for eighteen hours still contained 0.043 per cent of dextrose, the original percentage being however very high, 0.230. In another case (No. 26), in which the original percentage was normal all of the sugar had disappeared from the blood after twenty-four hours' incubation.

TABLE V.

TIME	PERCENTILE GLYCOLYSIS	NOS. OF EXPERIMENTS FROM WHICH FIGURES WERE TAKEN
30 min.....	12.9	9
40 min.....	14.2	16
1½ hrs.....	27.7	12
2 hrs.....	28.4	16
2½ hrs.....	44.3; 54.2	20; 7
2½ hrs.....	47.5; 50	9
3 hrs.....	42	12
4 hrs.....	64.5; 59	13; 16
4½ hrs.....	63.1; 81	4; 8
5 hrs.....	66.8; 79; 80.2	3; 17; 25
5½ hrs.....	76	15

*The blood constituent responsible for the glycolysis.*

In confirmation of the observations of others, we have found that no trace of sugar disappears from serum, at least after incubation for four hours. The agent responsible for glycolysis therefore resides in the corpuscles. In the deposit of centrifuged blood it was found that glycolysis is very rapid, all traces of sugar having disappeared from this in one experiment after ninety minutes' incubation. It was further found however that repeated washing of the corpuscular sediment with isotonic saline solution decidedly depresses the glycolytic power. Thus in two experiments, centrifuged corpuscles that had been repeatedly washed with isotonic saline and had meanwhile stood in this over night, were suspended in equal volumes either of isotonic saline or phosphate mixture or serum, sufficient dextrose being added in each

case to bring the percentage to about 0.3. As the following results show, it was found that very slight, if any glycolysis occurred, even although sterile conditions could not be strictly maintained.

*Experiment XXIII.*

1. 15 cc. washed corpuscles + 15 cc. serum + dextrose.  
     Per cent dextrose at start..... 0.324  
     Per cent dextrose after 5 hours' incubation .... 0.305
2. 15 cc. washed corpuscles + 7.5 cc. saline + 7.5 cc. phosphate mixture (2 pts.  $\text{NaH}_2\text{PO}_4$  + 8 pts.  $\text{Na}_2\text{HPO}_4$ , pH 7.4) + dextrose.  
     Per cent dextrose at start..... 0.330  
     Per cent dextrose after 5 hours' incubation .... 0.334
3. 15 cc. washed corpuscles + 15 cc. saline + dextrose.  
     Per cent dextrose at start..... 0.350  
     Per cent dextrose after 5 hours' incubation .... 0.338

*Experiment XXVI.*

1. 15 cc. washed corpuscles + 15 cc. serum previously incubated with glycogen.  
     Per cent dextrose at start..... 0.376  
     Per cent dextrose after 4 hours' incubation .... 0.332
2. 15 cc. washed corpuscles + 15 cc. serum + dextrose.  
     Per cent dextrose at start..... 0.474  
     Per cent dextrose after 4 hours' incubation .... 0.430

The absence or low degree of glycolysis in these experiments cannot be ascribed to there having been a deficiency of corpuscles in the suspensions, for in those of Experiment XXVI haemoglobin determinations by the Haldane method gave readings of 100 and 96 per cent. There may of course have been less haemoglobin in the other experiments but we scarcely think so.

The depression of glycolysis must therefore be due either to a deterioration of the glycolytic power as a result of standing or to a change in the permeability of the corpuscular envelope. Regarding the former possibility (that the glycolytic power itself had deteriorated) we have made observations on the glycolysis occurring in uncentrifuged blood that had stood for varying periods of time, either in the incubator or at room temperature. Table VI gives the results.

The glycolysis, as judged from the absolute amounts of dextrose that disappeared, was not distinctly less than in fresh blood in two of the observations in which the blood had stood in the

incubator for about twenty-four hours, but it was entirely absent in another blood that had stood for two days at room temperature. Since the corpuscular sediment in Experiments XXIII and XXVI had stood for less than twenty-four hours, and at the temperature of running water, we are inclined to attribute the loss of glycolytic power, which they exhibited, to the effect of washing.

It has been suggested (see p. 498) that glycolysis is an intracorporeal process, in other words, that before being destroyed in unlaked blood dextrose must be absorbed by the corpuscles. If this should prove to be the case, we may imagine that by the process of washing, the envelope becomes so altered that no

TABLE VI.

NO. OF EXP.	CONDITION OF BLOOD BEFORE ADDING DEXTROSE	TIME OF INCUBATION	PER CENT OF DEXTROSE IN BLOOD AT START	DEXTROSE DIS- APPEARED FROM 100 GMS. BLOOD
		<i>hours</i>		<i>grams</i>
19	Stood two days; sugar-free	3	0.519	0.001
26	Incubated 24 hrs.; sugar-free	4	a. 0.351 b. 0.655	0.056 0.072
21	Incubated over night; con- tained 0.043 per cent dextrose	4	0.329	0.075

absorption of dextrose can occur, hence no glycolysis. That such washing does alter the permeability of the envelope has been shown by Rona and Michaelis.<sup>15</sup> Laking of the blood does not however interfere with glycolysis, thus, in one experiment (31) the glycolysis in defibrinated blood amounted to 0.06 per cent dextrose in three and one-half hours: in the same blood laked by saponin, 0.073 per cent disappeared. In another experiment (32) 0.0405 per cent dextrose disappeared in three hours from saponin-laked blood.

Without further work it is impossible to say whether glycolysis is brought about by an action of the haemoglobin or of some

<sup>15</sup> Rona and Michaelis: *Biochem. Zeitschr.*, xvi, p. 60, 1909; *ibid.*, xviii, p. 375, 1909.

constituent of the envelope of the corpuscle. The fact that glycolysis can proceed in the absence of free oxygen (see p. 499) would tend to indicate that haemoglobin is not the responsible agent. In confirmation of this we found in one observation that the glycolytic process was not appreciably interfered with by the presence of coal gas (containing carbon monoxide) provided the flasks were kept in motion during the incubation. When the flasks were stationary however less sugar disappeared from the blood that was in contact with coal gas. Thus:

Original percentage of dextrose in blood.....	0.128
Gram dextrose disappeared in 3 hours from 100 grams blood in presence of air	
A. On shaker.....	0.096
B. Stationary.....	0.102
Gram dextrose disappeared in 3 hours from 100 grams blood in presence of coal gas	
A. On shaker.....	0.102
B. Stationary.....	0.074

*The relationship between glycolysis and the concentration and source of dextrose in the blood.*

One of the main objects of the present research was to see whether any evidence could be obtained indicating that the animal body does not utilize all varieties of dextrose with the same facility. It is a well established fact that the two varieties of dextrose  $\alpha$  and  $\beta$  do not exhibit the same behavior toward certain enzymes. It becomes a possibility therefore that the dextrose naturally present in blood which is produced by the enzymic hydrolysis of glycogen differs from commercial dextrose in the relative proportion of the two above varieties which it contains. We are not in possession of any facts that would justify us in concluding that there is really any difference between "commercial" and glycogen dextrose, but we have assumed that if such a difference should exist it might be indicated in the rate at which the two sugars disappear during glycolysis. It is of interest in this connection to note that Stoklasa<sup>16</sup> has claimed that hexoses are "fermented" by alcohol-ether preparations of pancreas juice

<sup>16</sup> Stoklasa: *Zeitschr. f. physiol. Chem.*, lxii, p. 35, 1909.

only when they have been prepared by enzyme hydrolysis. In order to study these questions we have made observations on:

1. Glycolysis in blood containing varying quantities of commercial dextrose.
2. Glycolysis in blood containing varying quantities of glycogen dextrose.

The glycogen dextrose was either prepared *in vitro* by incubating serum with glycogen or it was produced in the intact animal by inducing hyperglycaemia by means of one of the usual methods and then bleeding the animal.

### *1. The effect of adding commercial dextrose.*

In most of the experiments varying quantities of a sterile dextrose solution were added to fresh defibrinated blood, the rate of sugar disappearance being then determined in the original blood and in that containing the dextrose. The results are shown in Table VII.

The most important conclusion which may be drawn from these results is that the addition of dextrose to blood does not as a rule increase the amount of this substance which is destroyed when the blood is incubated for a given time. In other words, the absolute amount of dextrose which disappears is independent of the original concentration of this substance. This fact indicates that the glycolytic power of drawn blood must be very feeble, so feeble indeed that in a given time it can no more than deal with the normal amount of sugar present.

Although the rate of glycolysis cannot be increased by adding dextrose, yet if the incubation be prolonged, amounts of dextrose that are considerably in excess of the normal may ultimately be destroyed (Exp. No. 21). Instead of increasing the glycolysis, the addition of large quantities may slightly depress it, as is shown in case of experiment 16, 22 and X of Table VII. This observation raises the question as to the propriety of adding dextrose to the blood, or other nutritive fluid, employed for perfusing the heart, or other tissues, in order to study sugar utilization. By such additions no other advantage can be gained than that less fluid will have to be taken for analysis while, on the other hand, apart from the possible depression in glycolysis in



TABLE VII.

NO. OF EXP.	TIME OF INCUBATION	BLOOD ALONE		BLOOD + DEXTROSE	
		Per cent dextrose in blood at start	Gms. dextrose disappeared from 100 gms. blood	Per cent dextrose in blood at start	Gms. dextrose disappeared from 100 gms. blood
16	40 min.	0.176	0.025	0.524	0.014
	2 hrs. 10 min.		0.050		0.051
	4 hrs. 10 min.		0.104		0.066
20	1 hr.	0.230	0.025	0.465	
	3 hrs.		0.045		0.070
	5 hrs.		0.094		0.116
21	1 hr.	0.043 (after 24 hrs.' in- cubation.)		0.329	0.039
	3 hrs.				0.054
	4 hrs.				0.075
	20 hrs.				0.257
22	2½ hrs.	0.106	0.047 0.106(?)	A. 0.436	0.031
	4½ hrs.			B. 0.840	0.116
	2½ hrs.			C. 1.139	0.087
	4½ hrs.				0.093
	4½ hrs.				0.093
24	2 hrs.	0.173	0.029	A. 0.329	0.032
	2 hrs.			B. 0.475	0.060
	4 hrs.			C. 0.973	0.072
X	2 hrs.	0.150	0.124	0.351	0.031
	5 hrs.				0.111
26	4 hrs.	none (incu- bated 48 hrs.)		A. 0.351	0.056
	4 hrs.			B. 0.665	0.072

the blood itself, the risk is incurred that the excess of dextrose exercises a depressing influence on tissue glycolysis, or it may encourage a deposition of dextrose in a condensed form within the tissues.

Before proceeding to discuss the bearing of these facts on the possible importance of blood glycolysis in the utilization of carbohydrate in the animal body we shall consider the influence produced on glycolysis by adding glycogen dextrose to blood.

In one observation 15 cc. of sterile serum were incubated with 0.1 gram of glycogen for one hour by which time, as control experiments showed, hydrolysis was complete. The resulting serum was then shaken with 15 cc. of centrifuged corpuscles, the percentage of haemoglobin in the mixture being 100. Three quantities of 10 cc. each were then incubated and gave 0.376 per cent dextrose to start, 0.357 after two hours' incubation and 0.332 per cent after four hours, or 0.019 gram dextrose disappeared from 100 grams of blood in two hours and 0.044 in four hours. The above figures, in a control observation with the same serum and corpuscle mixture but containing added commercial dextrose were: 0.474 (start), 0.441 (2 hours), 0.430 (4 hours) or 0.033 gram dextrose disappeared in two hours and 0.044 in four hours. The glycogen dextrose was therefore destroyed at the same rate as commercial dextrose.

However carefully such experiments might be controlled, the objection could always be raised that the manipulations involved in them had altered the glycolytic power of the corpuscles. Experiments were therefore undertaken in which glycolysis was studied in blood removed from hyperglycaemic animals. The hyperglycaemia was induced in some cases by stimulation of the great splanchnic nerve and in others, by curare or adrenalin. In the splanchnic nerve experiments the blood was usually removed after ten minutes' stimulation so that the hyperglycaemia was of slight degree. In the case of the asphyxial and adrenalin experiments it was removed much later so that marked hyperglycaemia existed. In all the experiments before inducing the hyperglycaemia some blood was removed in order that the normal rate of glycolysis might be compared with that occurring in hyperglycaemic blood. The results are shown in Table VIII.

It is very definitely shown, especially by the adrenalin experi-

EXPT. NO.	TIME OF INCUBATION	NORMAL BLOOD			DIABETIC BLOOD			REMARKS
		Per cent dextrose to start	Dextrose disappeared from 100 gms. blood	grams	Method used for producing hyperglycaemia	Per cent dextrose to start	Dextrose disappeared from 100 gms. blood	grams
13	2 hrs.	0.126	0.022	0.00018	Stim. splanchnic nerve for 10 min.	0.148	0.026	0.00022
	4 hrs.		0.081	0.00034			0.089	0.00037
14	1 hr.	0.172	0.013	0.00043	Stim. splanchnic nerve for 12 min.	0.205	0.009	0.0003
	4½ hrs.		0.076	0.00028			0.079	0.00029
16	40 min.	0.176*	0.025	0.0006	Stim. splanchnic nerve for 30 min.	0.200†	0.017	0.00042
	4 hrs.		0.104	0.00043			0.062	0.00025
27	2 hrs.	0.120	0.037	0.0003	Curare injected but respiratory movements persisted.	0.161	0.014	
	5 hrs.		0.081	0.00027				Femoral artery blood.
29	2 hrs.	0.088	0.051	0.0004	Adrenalin (10 cc. 1-1000) injections for 1½ hrs.	0.454	0.058	0.00041
	4 hrs.		0.088†				0.078	0.00032
30	30 min.	0.223§			Adrenalin injections for 1 hr. 20 min.	0.502	0.019	0.00063
	1 hr.		0.022	0.00037			0.045	0.00034
	2 hrs.		0.073	0.00030			0.077	0.00030

\* Vena cava blood.

† Femoral artery blood.

‡ I.e., all sugar disappeared.

§ Blood very venous.

ments, that even a very great increase in the natural (glycogen) dextrose of the blood does not cause any more than the usual amount of this substance to be destroyed during *in vitro* incubation. There is therefore no apparent difference between the two kinds of dextrose in so far as their destructibility by means of blood is concerned. The glycolytic power of the blood for both commercial and for glycogen dextrose is strictly limited nor does it become appreciably altered in at least two of the varieties of hyperglycaemia (splanchnic stimulation and adrenalin).

From these as well as the foregoing results it is plain that the *glycolysis which we study in blood in vitro does not bear any important relationship to the glycolysis which occurs in the intact animal*. The amount of sugar which disappears from blood *in vitro* is by far too small to account for more than a minute fraction of that which disappears in the body.

Thus, in eviscerated dogs we have found<sup>17</sup> that from 0.83 mgm. to 4.46 mgm. dextrose disappear from 100 grams of blood per minute. Whereas these values for blood *in vitro*, as the above observations show, only range between 0.03 and 0.06 mgm., even during the early stages of the process when it is most rapid. Similar calculation of the results given in the other tables yields corresponding values. These differences between the glycolysis of blood and intact tissues cannot be explained as due to the ready destructibility of the glycolytic agent outside the body for, if this were so, we should expect the glycolytic power to fall off rapidly after the blood is drawn, which however we have seen is by no means the case.

The absence of glycolytic power in the serum and the other facts which make it extremely probable that dextrose must be absorbed into the corpuscles before it can be destroyed further indicate the unimportance of blood glycolysis in the intact animal. Nor does it appear that the intracorpuseular glycolysis can even be made use of to get rid of excessive amounts of circulating dextrose, and thus prevent hyperglycaemia, for we have seen that the process does not become more active under such conditions. Furthermore, the practical constancy in the glycolytic power of the blood before and after an animal is rendered hyperglycaemic

<sup>17</sup> Macleod and Pearce: *Amer. Journ. of Physiol.*, xxxii, p. 184, 1913.

shows that alterations in this process can play no rôle in the cause of the hyperglycaemia.

*The supposed existence of "sucre virtuel" in freshly drawn blood.*

Lépine and Barral<sup>18</sup> have claimed that the concentration of actual sugar may be greater in blood that has stood for from fifteen minutes to an hour at body temperature outside the body, than in freshly drawn blood. So far as we are aware, no subsequent worker has directly tested this claim.

TABLE IX.

NO. OF EXP.	PER CENT SUGAR IN BLOOD IMMEDI- ATELY AFTER COLLECTING	PER CENT SUGAR IN SAME BLOOD AFTER STANDING	TIME OF STANDING	REMARKS
			min.	
5	0.107	0.101		This blood stood for some time in ice-cold water before precipitating.
6	{ 0.131 0.135	0.129	15	
7	{ 0.154 0.154	0.129	40	
11	0.207	0.202	20	
13	0.145	0.136	20	From femoral artery.
	0.161	0.148	20	From vena cava.
14	0.172	0.159	30	From femoral artery.
	0.205	0.176	30	From vena cava.
14a	0.205	0.196	30	From femoral artery.

In order to do so, we have compared the sugar in blood collected in ice-cold water and then immediately precipitated with colloidal iron, with that of blood collected either immediately before or afterwards (practically simultaneous) but allowed to stand before precipitation in the incubator for varying periods up to one hour. The results, which are collected in Table IX, show that there was always less sugar in the incubated blood up to 30 minutes after removal. That the decrease becomes more marked after longer periods has already been shown in the previous tables.

<sup>18</sup> Lépine: *Le diabète sucré*, Paris (Felix Alcan), 1909, p. 64.

On account of the absence of any evidence for the supposed increase in sugar, we have in most of our later experiments collected the control blood in water and precipitated it immediately.

#### CONCLUSIONS.

1. Unclothed (hirudin) and defibrinated blood have the same glycolytic power, but potassium oxalate, in concentrations of one per thousand and over, has a depressing action.

2. The rate of glycolysis varies from time to time in the defibrinated blood of the same animal. It therefore varies also in the blood of different animals of the same species.

3. On an average, about one-half of the original amount of dextrose disappears in two and one-half hours from blood kept outside the body at 40°C.

4. Glycolysis is a function of the corpuscles and is absent in the serum. It disappears from the corpuscles after frequent washing with isotonic saline.

5. The addition of dextrose to blood does not materially increase the extent of the glycolysis occurring in a given time. In higher concentrations it may indeed depress the process.

6. The source of the dextrose, *i.e.*, whether chemical or derived from glycogen by the action of glycogenase, bears no relationship to the rate of glycolysis. Glycolysis proceeds at the same rate in normal as in "diabetic" blood.

7. Even under the most favorable circumstances the quantity of dextrose which the blood can destroy is only a small fraction of that which disappears in the same time in the intact animal. The glycolysis which occurs in blood is most probably of no importance in carbohydrate metabolism.

8. No evidence could be obtained of an increase of sugar as a result of allowing freshly drawn blood to stand at body temperature for varying periods of time up to one hour.

## RESEARCHES ON PURINES. XII.<sup>1</sup>

### ON 2-OXY-6-METHYL-9-ETHYLPURINE, 2-OXY-6,8-DIMETHYL-9-ETHYLPURINE, 2-OXY-6-METHYL-8-THIO-9-ETHYLPURINE, 2-OXY-6-METHYL-9-ETHYLPURINE-8-THIOGLYCOLLIC ACID, AND 2-METHYLMERCAPTO-6-OXY-8-THIOPURINE.

By CARL O. JOHNS AND EMIL J. BAUMANN.

(From the Sheffield Laboratory of Yale University.)

(Received for publication July 31, 1913.)

In the eleventh paper of this series we stated that 2-oxy-4-methyl-5-amino-6-ethyl-aminopyrimidine (II)<sup>2</sup> reacted readily, when heated with urea, to form 2,8-dioxy-6-methyl-9-ethylpurine (VI).<sup>3</sup> We find that this pyrimidine also reacts smoothly with other reagents which are commonly used for the preparation of purines from orthodiaminopyrimidines.

Thus, when the formyl derivative of 2-oxy-4-methyl-5-amino-6-ethylamino pyrimidine is heated, water is liberated and 2-oxy-6-methyl-9-ethylpurine (I) is formed. When the corresponding acetyl compound was heated we obtained 2-oxy-6,8-dimethyl-9-ethylpurine (III). The yields in both cases were excellent. This diaminopyrimidine also reacts normally when heated with thiourea and forms 2-oxy-6-methyl-8-thio-9-ethylpurine (IV). The yield was 61 per cent of the calculated. The thiopurine, thus obtained, reacted with monochloracetic acid and gave 2-oxy-6-methyl-9-ethylpurine-8-thioglycollic acid (V). This compound is stable in hot water but when boiled with concentrated hydrochloric acid it is hydrolyzed to 2,8-dioxy-6-methyl-9-ethylpurine (VI). The thioglycollic acid derivative forms a crystalline ammonium salt. We have also investigated the action of thiophosgene on 2-methyl-

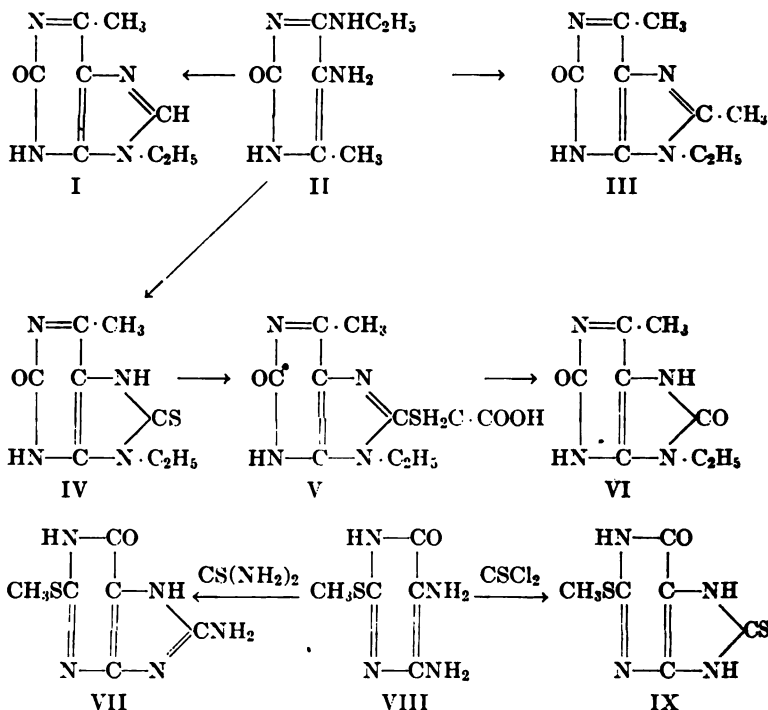
<sup>1</sup> Johns and Baumann: this *Journal*, xv, p. 119, 1913.

<sup>2</sup> Johns and Baumann: *ibid*, xv, p. 123, 1913.

<sup>3</sup> Johns and Baumann: *ibid*, xv, p. 124, 1913.

mercapto-4,5-diamino-6-oxy-pyrimidine (VIII)<sup>4</sup> and find that 2-methylmercapto-6-oxy-8-thiopurine (IX) is formed. The yield was low, about 44 per cent of the calculated, but a suitable solvent for the diaminopyrimidine was not found and the reaction was carried out in the presence of water which decomposed the thiophosgene to a considerable extent. This formation of 2-methylmercapto-6-oxy-8-thiopurine is of interest since we found previously that when thiourea and 2-methylmercapto-4,5-diamino-6-oxy-pyrimidine were heated together the resulting compound was 2-methylmercapto-6-oxy-8-aminopurine (VII)<sup>5</sup> instead of the expected 2-methylmercapto-6-oxy-8-thiopurine (IX).

These researches will be continued.

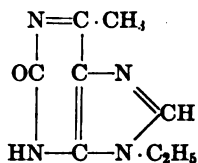


<sup>4</sup> Johnson, Johns and Heyl: *Amer. Chem. Journ.*, xxxvi, p. 172, 1906.  
Johns and Baumann: this *Journal*, xiv, p. 385, 1913.

<sup>5</sup> Johns and Baumann: this *Journal*, xiv, p. 387, 1913.



## EXPERIMENTAL PART.

*2-Oxy-6-methyl-9-ethylpurine.*

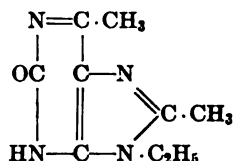
Three grams of 2-oxy-4-methyl-5-amino-6-ethyl-aminopyrimidine<sup>6</sup> were dissolved in 15 cc. of 85 per cent formic acid. Solution took place at room temperature and considerable heat was evolved. This solution was then boiled gently under a return condenser for fifteen minutes and was finally evaporated to dryness on a steam bath. The residue was heated in an air bath at 170°–180°C. for an hour. The reaction product was dissolved in hot water and clarified once with blood coal. This treatment left a colorless solution which was concentrated to a small volume and allowed to cool slowly. 2-Oxy-6-methyl-9-ethylpurine crystallized out in needles which formed a network with a silky appearance. A second crop of crystals was obtained by concentrating the mother liquor. The yield was nearly quantitative. This purine did not contain water of crystallization after it had been dried over sulphuric acid for three days. It began to melt with partial decomposition at about 256°C. and at 275°C. all had melted to a dark oil. It was very soluble in hot water or alcohol as well as in cold dilute acids and alkalies. It did not dissolve in ether and was but slightly soluble in benzene. An aqueous solution of the purine did not give a precipitate with barium chloride or picric acid but gave a white precipitate with mercuric chloride. This was soluble in hot water and separated again on cooling. The purine gave a white gelatinous precipitate with ammoniacal silver. Nitric acid oxidized the purine readily and on evaporation a yellow crust remained. This turned red when it was moistened with a drop of alkali and dried on the steam bath.

<sup>6</sup> Johns and Baumann: this *Journal*, xv, p. 123, 1913.

0.1448 gram of substance gave 0.2871 gram of  $\text{CO}_2$  and 0.0708 gram of  $\text{H}_2\text{O}$ .

	Calculated for $\text{C}_7\text{H}_{10}\text{ON}_4$ :	Found:
C.....	53.93	54.07
H.....	5.62	5.46
N.....	31.46	31.51

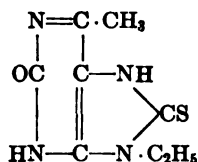
*2-Oxy-6,8-dimethyl-9-ethylpurine.*



Three grams of 2-oxy-4-methyl-5-amino-6-ethylaminopyrimidine were dissolved in 15 cc. of acetic anhydride and the solution was boiled for five minutes after which it was evaporated to dryness on a steam bath. The residue was then heated in an air bath at  $180^\circ\text{C}$ . for an hour. It was then dissolved in water and the solution was decolorized with blood coal. On concentrating to a small volume and cooling slowly a network of silky needles was obtained. These needles were found to be anhydrous after drying over sulphuric acid for three days. The yield was almost theoretical. The crystals began to shrink at about  $230^\circ\text{C}$ . and melted to a dark oil at about  $265^\circ\text{C}$ . This purine was very soluble in hot water or alcohol and also in cold dilute acids and alkalis. It was not soluble in ether and only slightly soluble in benzene. Its aqueous solution did not give a precipitate with barium chloride or picric acid but gave white precipitates with solutions of mercuric chloride and ammoniacal silver. Nitric acid attacked the purine readily and when the resulting solution was dried a yellow residue remained. This turned red when moistened with ammonia.

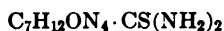
0.2098 gram of substance gave 0.4313 gram of  $\text{CO}_2$  and 0.1176 gram of  $\text{H}_2\text{O}$ .

	Calculated for $\text{C}_8\text{H}_{12}\text{ON}_4$ :	Found:
C.....	56.25	56.07
H.....	6.25	6.23
N.....	29.17	29.26

*2-Oxy-6-methyl-8-thio-9-ethylpurine.*

Six grams of 2-oxy-4-methyl-5-amino-6-ethylaminopyrimidine and an equal weight of thiourea were pulverized together and the mixture was heated for an hour at 175° to 185°C. in an oil bath. As the thiourea melted the mass darkened, frothing occurred and in about a half hour the whole mass solidified. The reaction product was dissolved in hot dilute ammonia and after clarifying with blood coal the solution was acidified with acetic acid. The purine was thrown down at once from the hot solution. It was purified by dissolving in hot dilute ammonia and precipitating with acetic acid. After drying at 100°C. the yield was 4.2 grams or 61 per cent of the calculated weight. The substance, thus obtained, decomposed at 295° to 300°C. It was difficultly soluble in hot water and on cooling the solution slowly it crystallized in sheaves. It was slightly soluble in hot alcohol but did not dissolve in benzene or ether. It dissolved readily in dilute acids or alkalies. Its aqueous solution did not give a precipitate with barium chloride or picric acid. It formed insoluble compounds with mercuric chloride and ammoniacal silver solutions. Nitric acid oxidized it readily, leaving a yellow residue on evaporation. This turned red where moistened with ammonia.

	Calculated for $\text{C}_8\text{H}_{10}\text{ON}_4\text{S}$ :	Found:	
		I	II
N.....	26.67	26.92	26.79

*The thiourea addition product of 2-oxy-4-methyl-5-amino-6-ethylaminopyrimidine.*

Six-tenths gram of 2-oxy-4-methyl-5-amino-6-ethylaminopyrimidine were dissolved in a little hot water and an equal weight of thiourea was added. The thiourea dissolved, reaction took place and a solid substance separated rapidly. After cooling, the

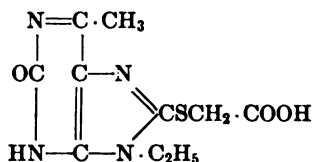
solid was filtered off and washed with a little cold water. It was rather soluble in hot water and sparingly soluble in cold water and but slightly soluble in alcohol. The crystals melted to a dark oil at 204°–206°C.

	Calculated for $C_8H_{10}ON_4S$ :	Found:
N.....	34.43	34.38

When the above compound was heated at 175°–185°C. ammonia was evolved, frothing and darkening took place and a solid residue was formed. When this product was dissolved in dilute ammonia, the solution clarified with blood coal and acidified with acetic acid, a precipitate of 2-oxy-6-methyl-8-thio-9-ethylpurine resulted.

	Calculated for $C_8H_{10}ON_4S$ :	Found:
N.....	26.67	26.79

*2-Oxy-6-methyl-9-ethylpurine-8-thioglycollic acid.*



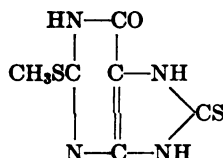
Two grams of 2-oxy-6-methyl-8-thio-9-ethylpurine were suspended in 150 cc. of water containing 10 grams of monochloroacetic acid and the mixture was boiled under a return condenser until the purine had dissolved. This required about an hour. After filtering to remove a slight turbidity the solution was cooled, whereupon a bulky mass of needles crystallized out. A second crop was obtained by concentrating the mother liquor. The yield was 1.7 grams or 65 per cent of the theoretical amount. The thioglycollic acid derivative was readily soluble in hot and moderately soluble in cold water. It was slightly soluble in hot alcohol and not soluble in benzene. It was moderately soluble in hot glacial acetic acid and dissolved readily in dilute mineral acids or alkalies. It dissolved in hot dilute ammonia and on cooling the solution hair-like crystals were obtained. It began to darken at 270°C. and decomposed gradually at higher temperatures.

	Calculated for $C_{10}H_{12}O_2N_4S$ :	Found:
N.....	20.90	20.99

*Hydrolysis of 2-oxy-6-methyl-9-ethylpurine-8-thioglycollic acid.*

When this thioglycollic acid derivative was heated with concentrated hydrochloric acid under a reflux condenser for two hours hydrolysis occurred. The resulting solution was evaporated to dryness and the residue was dissolved in ammonia and this solution was acidified with acetic acid. The compound thus obtained crystallized in sheaves and exhibited all the properties of 2,8-dioxy-6-methyl-9-ethylpurine. It was free from sulphur and did not melt or decompose at 320°C.

	Calculated for $C_8H_{10}O_2N_4$ :	Found:
N.....	28.87	28.93

*2-Methylmercapto-6-oxy-8-thiopurine.*

Five grams of 2-methylmercapto-6-oxy-4,5-diaminopyrimidine<sup>7</sup> were dissolved in boiling water and the solution was cooled rapidly in order to obtain small crystals. Fifteen grams of thiophosgene were added gradually with thorough shaking. This operation was carried out in a glass stoppered bottle. The solvent became blue and a granular substance separated. This was filtered off, dissolved in hot dilute ammonia and reprecipitated with acetic acid. It crystallized in small globules which were soluble in about 200 parts of boiling water and almost insoluble in cold water. They did not dissolve in ether and were but slightly soluble in hot alcohol or glacial acetic acid. They began to decompose at about 275°C. They gave the murexide test. The yield was low since a suitable solvent for the diaminopyrimidine was not found and water decomposed the thiophosgene. The amount obtained from 5 grams of the diaminopyrimidine was only 1.5 grams. However, 1.6 grams of the diaminopyrimidine were recovered by evaporating the mother liquor thus making the yield of thiopurine about 44 per cent of the theoretical amount.

	Calculated for $C_8H_8ON_4S_2$ :	Found:	
		I	II
N.....	26.17	26.13	26.16

<sup>7</sup> Johns and Baumann: this *Journal*, xiv, p. 385, 1913.



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## THE PARTIAL PURIFICATION OF THE ESTERASE IN PIG'S LIVER.

By GEORGE PEIRCE.

*(From the Physiological Laboratory of the University of Wisconsin.)*

(Received for publication, August 1, 1913.)

The preparation of a substance which can be regarded as an enzyme free from foreign material has never been accomplished. Invertases and proteases of considerable strength have been prepared, but very little work has been done on the purification of lipases. The preliminary report of work with the esterase from pig's liver may therefore be of interest.

The crude enzyme solution was prepared in much the same manner as in previous investigations. One hundred grams of fresh pig's liver were ground with sand and water, strained through cloth and made up to 1 liter with distilled water. Toluene was added as a preservative. After incubation at 37° for one day and after several weeks' standing at room temperature it was filtered through a folded filter until clear. The filtrate will be referred to as 10 per cent crude enzyme solution. 20 per cent solutions were also made.

The crude enzyme solution was dialyzed in collodion bags for five or six days and filtered. About 90 per cent of the solid substance was removed by this process, and the solution lost about 20 per cent of its total activity so that the purification was considerable. This solution will be referred to as dialyzed enzyme solution.

In it was now dissolved one-half the amount of ammonium sulphate necessary for complete saturation, and the solution was poured repeatedly through the same folded filter until a clear filtrate was obtained. The precipitate was practically inactive and was rejected. The filtrate was then fully saturated with ammonium sulphate and filtered till clear. The filtrate was inactive. The precipitate was taken up in water and the solution dialyzed till it no longer gave a turbidity with  $\text{BaCl}_2$ . This

represents the most highly purified solution obtained (Solution B). No attempt was made to get a solid active substance.

A small portion of one of these solutions was mixed with an ethyl butyrate solution about two-thirds saturated. Both solutions had been previously warmed to 37° and the reaction was carried on at this temperature. The time of the beginning of the reaction (which was not more than three seconds in error) was taken at the time when one-half of the enzyme had flowed from a pipette into the ethyl butyrate solution. At suitable intervals 50 cc. were removed with a pipette and allowed to flow into an Erlenmeyer flask containing 5 cc. of 1 per cent neutralized NaF. The mixture was titrated with  $\frac{N}{10}$  NaOH free from carbonate to a moderately deep shade of pink, phenolphthalein being used as the indicator. NaF of the above strength inhibits the enzyme completely in acid solutions, but allows the hydrolysis to proceed slowly in neutral or faintly alkaline solutions. Consequently the titrations need not be made immediately, but the final stage must be performed in a few seconds. This was one of the reasons for the selection of a deeper shade of pink than usual for an end point. The time of completion of the reaction is taken when the first drop from the pipette flows into the NaF. This allows for the slowing of the hydrolysis by cooling during the pipetting and for progress of the reaction in that part of the reaction mixture that has not yet been mixed with the NaF. The error in the measurement of the time was probably less than ten seconds in all. The titration error probably averaged about 0.1 cc. or a little less. Proper allowance was always made for any initial acidity. The temperature regulation was efficient and no error is to be attributed to this source.

The activities of the enzyme solutions are represented on the basis of their solid content. It has been previously shown<sup>1</sup> that in solutions of equal acidity, a given amount of enzyme hydrolyzes ethyl butyrate with the same absolute velocity over a wide range of enzyme and ester concentration. Consequently it will be convenient for the present purpose to compare the activities of the different solutions by giving the number of parts of ethyl butyrate that one part of solid substance hydrolyzes per hour. The ethyl butyrate concentration must always be above  $\frac{N}{10}$  and

<sup>1</sup> Peirce: *Journ. Amer. Chem. Soc.*, xxxii, pp. 1525 and 1530, 1910.

the acidity must be the same in the solutions to be compared. In this paper it will be understood that the initial acidity is "0" and the final acidity "10," i.e., 10 cc. of  $\frac{N}{10}$  acid per 50 cc. of reaction mixture.

15 cc. of Solution B contained 4.8 mgms. solid substance (dried over water bath).

20 cc. of this solution in a total volume of 560 cc. hydrolyzed 650 mgms. ethyl butyrate in 28.1 minutes (final acidity "10").

The activities of the various solutions follow:

DESIGNATION OF ENZYME SOLUTION	PARTS OF ETHYL BUTYRATE HYDROLYZED PER HOUR BY 1 PART SOLID SUBSTANCE
A <sub>1</sub> . Crude 10 per cent.....	10*
A <sub>2</sub> . Dialyzed 10 per cent.....	90
A <sub>3</sub> . Partially† purified 10 per cent.....	165
B. Purified 20 per cent.....	217

\* Initial acidity 1.4, final acidity 11.4.

† The half saturation with ammonium sulphate was omitted.

Kastle, Johnston and Elvove<sup>2</sup> prepared a clear esterase solution and estimated its activity on the basis of its solid content. Without any allowance for the different conditions of the hydrolysis, the solid substance in solution B was 400 times as active as that in their solution. Making a liberal allowance for differences in the conditions it seems safe to say that the solid substance of solution B was 50 and probably 100 times as active as theirs.

No detailed chemical investigation was made of the dried material from these enzyme solutions, owing to the small amount of material available. A<sub>3</sub> contained about 0.6 per cent phosphorus, but the figure is only approximate. An accurate determination of the tyrosine content of B was made. 4.8 mgms. of the dried substance were heated on a water bath for several hours with 20 per cent HCl, the HCl was evaporated and the residue taken up with a few drops of dilute HCl. A determination according to Folin, using one-quarter of the given quantity of reagents and diluting to 25 cc., gave the following result:

4.8 mgms. dried substance gave 0.283 mgm. tyrosine. Tyrosine=5.9 per cent.

<sup>2</sup> *Amer. Chem. Journ.*, xxxi, p. 526, 1904.



## THE COMPOUND FORMED BETWEEN ESTERASE AND SODIUM FLUORIDE.

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In previous papers by Kastle and Loevenhart<sup>1</sup> and Loevenhart and Peirce<sup>2</sup> it was shown that sodium fluoride has a remarkable inhibiting action on lipases and esterases. In the present paper it is proposed to show: (1) that this inhibition is due to the formation of an inactive compound composed wholly, or in part, of sodium fluoride and the enzyme; (2) that the reaction in which this compound is formed, is reversible; (3) that an equation for the reaction based on the mass law, agrees with the observations.

The particular case investigated was that of the action of the esterase from pig's liver on ethyl butyrate. The choice was made on account of ease of experimentation and it is planned later to extend the work to other simple esters, and, if possible, to the true fats. Four different enzyme solutions were used. For the methods of their preparation the previous paper in this number of the *Journal* must be consulted. Preparation A was the crude 10 per cent enzyme there referred to, while B was the most highly purified enzyme obtained in that investigation. The crude 10 per cent enzyme solution used in Table III was about one-third as active as A. C was prepared in the same manner as B but was not quite so active.

The technique was the same as that of the previous paper except in Table V, where alcohol instead of sodium fluoride was used to stop the reaction before titration. When sodium fluoride was used in a reaction mixture it was mixed with the ethyl butyrate before the enzyme was added.

<sup>1</sup>*Ver. Chem. Journ.*, xxiv, p. 491, 1900.

<sup>2</sup>*Journal*, ii, p. 397, 1907.

It has been shown<sup>3</sup> that the concentration of ethyl butyrate makes practically no difference in the absolute velocity of acid production when no sodium fluoride is present. This has not been shown to be true in sodium fluoride mixtures, so to obviate any difficulty the initial concentration of ethyl butyrate is the same in any given set of experiments. It varied from  $\frac{N}{25}$  to  $\frac{N}{10}$  in different sets of experiments.

Using enzyme solution B several series were run, alike in all respects except that the concentration of the sodium fluoride varied. The numerical data for this series of experiments are given in Table I, and the results shown graphically in Figure 1.  $x$  is the

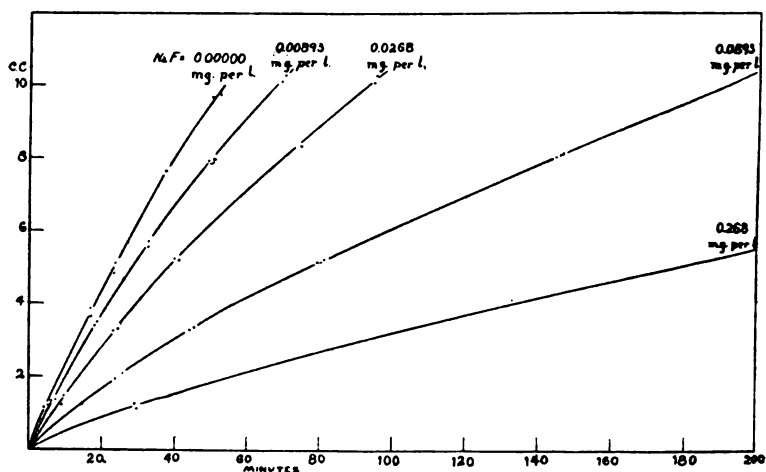


FIG. 1. TO ILLUSTRATE TABLE I.

number of cc. of  $\frac{N}{25}$  butyric acid produced in 50 cc. of reaction mixture in  $t$  minutes. If the decimal point is moved two places to the left, the normality of the solution is obtained. The time taken to produce 10 cc. of acid is calculated for each series and reappears in the third column of Table II. The reaction mixtures were made up as follows:

- 250 cc. ethyl butyrate solution.
- 5 cc. enzyme solution B.
- 25 cc. { sodium fluoride solution of different strengths.
- water.

<sup>3</sup> Peirce: *Journ. Amer. Chem. Soc.*, xxxii, p. 1525, 1910.



TABLE I.

NaF = 0.00000 MG. PER LITER				NaF = 0.00893 MG. PER LITER			
Observed		Average		Observed		Average	
<i>z</i>	<i>t</i>	<i>z</i>	<i>t</i>	<i>z</i>	<i>t</i>	<i>z</i>	<i>t</i>
1.22	5.22	1.18	5.03	1.29	5.92	1.32	6.52
1.14	4.83			1.34	6.92		
3.62	16.92	3.73	17.02	3.48	18.17	3.40	18.00
3.84	17.12			3.31	17.83		
4.84	23.17	4.97	23.56	5.65	32.75	5.62	32.63
5.10	23.95			5.58	32.50		
7.62	37.42	7.64	37.52	7.92	49.30	7.96	49.69
7.65	37.62			7.99	50.08		
9.64	50.55	9.69	51.43			(10.00)	(67.8)
9.73	52.30	(10.00)	(53.7)	10.10	69.00		
					70.08	10.19	69.54
				10.27			

NaF = 0.0268 MG. PER LITER				NaF = 0.0893 MG. PER LITER			
Observed		Average		Observed		Average	
<i>z</i>	<i>t</i>	<i>z</i>	<i>t</i>	<i>z</i>	<i>t</i>	<i>z</i>	<i>t</i>
1.22	8.77	1.33	9.11	2.09	25.70	2.01	24.69
1.44	9.45			1.92	23.67		
3.21	22.97	3.26	23.55	3.34	44.92	3.30	44.46
3.31	24.12			3.26	44.00		
5.12	39.85	5.12	40.28	5.21	80.88	5.16	79.78
5.12	40.70			5.10	78.67		
8.33	74.47	8.33	74.38	8.15	146.78	8.08	145.56
8.32	74.28			8.01	144.33		
		(10.00)	(92.8)			(10.00)	(190. )
10.09	94.28	10.18	94.83	10.52	202.83	10.47	201.42
10.26	95.37			10.42	200.00		

NaF = 0.268 MG. PER LITER			
Observed		Average	
<i>z</i>	<i>t</i>	<i>z</i>	<i>t</i>
1.14	29.08	1.17	29.04
1.20	29.00		
3.85	133.58	4.00	133.21
4.15	132.83		
5.65	212.83	5.70	213.23
5.75	213.62		
7.96	338.08	8.01	338.29
8.06	338.50		
		(10.00)	(450. )
10.39	479.42	10.51	479.77
10.62	479.92		

The most obvious explanation for this inhibition is that a certain amount of the enzyme is inactivated by the sodium fluoride, either by destruction, or by the formation of some sort of inactive compound. We shall see later that the view that the enzyme is destroyed is untenable.

Any compound of sodium fluoride and esterase should be formed in accordance with the mass law:

$$(\text{Conc. free Enz.})^m \times (\text{conc. free NaF})^n = k (\text{conc. NaF. Enz.})^p \dots [1]$$

where  $m$ ,  $n$  and  $p$  represent the number of molecules of the substances involved in the reaction.

Transposing:

$$k = \frac{(\text{conc. free Enz.})^m}{(\text{conc. NaF. Enz.})^p} \times (\text{conc. free NaF})^n \dots [2]$$

We have no data at present for assigning values to  $m$ ,  $n$  and  $p$ , but a little consideration will enable us to exclude several possibilities at the outset and leave only a few others to be tested. It will be shown experimentally in Table V that the formation of the inactive compound is reversible. It is difficult to conceive of a reaction, wherein one molecule of active enzyme would be broken up by sodium fluoride into two or more molecules of an inactive compound, in such a manner that the reaction could be reversible. This would indicate that in equations [1] and [2]  $m$  is equal to or greater than  $p$ . For simplicity we can let  $p = 1$  and  $m = 1, 2, 3$  or more. The higher values are, of course, increasingly improbable. No values can be assigned to  $n$  on such considerations as these.

On the basis of the figures already obtained, the most simple equation possible will first be tested:

$$k = \frac{\text{conc. free Enz.}}{\text{conc. NaF Enz.}} \times \text{conc. free NaF} \dots [3]$$

That is, we assume that one molecule of sodium fluoride combines with one molecule of enzyme to form one molecule of the inactive compound. The term  $\frac{\text{conc. free Enz.}}{\text{conc. NaF. Enz.}}$  is calculated as follows:

It has been shown repeatedly<sup>4</sup> that the absolute velocity of acid

<sup>4</sup> Cf. Peirce: *Journ. Amer. Chem. Soc.*, xxxii, p. 1529, 1910.

production is very nearly proportional to the amount of enzyme present. In any event, a solution whose activity is inhibited by sodium fluoride behaves as if only a certain percentage of the total enzyme present were really acting. Let  $x$  represent this percentage. Then since the total percentage present is 100, the percentage of the NaF. Enz. present is  $100 - x$  and  $\frac{\text{conc. free Enz.}}{\text{conc. NaF. Enz.}} =$

$\frac{x}{100 - x}$ . For example it took a solution containing no sodium fluoride 60 minutes to produce 10 cc. of  $\frac{N}{10}$  acid, and a solution containing 1:40,000,000 sodium fluoride 100 minutes to produce the same amount. The one containing sodium fluoride had therefore  $\frac{60}{100} = 60$  per cent of the activity of the other. If the activity as measured in this way is proportional to the active enzyme present, we may say that 60 per cent is "free" and 40 per cent combined with sodium fluoride. The ratio<sup>5</sup>  $\frac{\text{conc. free Enz.}}{\text{conc. NaF. Enz.}}$  is

<sup>5</sup> The velocity falls off during the reaction so that the activity as measured by the reciprocal of the time taken to attain a given stage of the reaction represents the average activity during this period. Since the curves with different strengths of sodium fluoride are not exactly similar in shape a slight inaccuracy is involved. Absolutely accurate results can theoretically be obtained by plotting the curves with  $x$  and  $t$  as ordinate and abscissa respectively, and taking the values of the tangents  $\frac{dx}{dt}$  at the same values of  $x$  on the different curves as proportional to the values of the activities at these points. Since now the curves have a nearly constant curvature, the value of  $\frac{x}{t}$  for  $x = 10$  will be very nearly equal to  $\frac{dx}{dt}$  for  $x = 5$ . The simpler procedure is used for two reasons. First, the measurements are not sufficiently accurate to justify the labor involved in making an extremely accurate graphical measurement and, secondly, the method employed is absolutely objective.

Expressed as non-mathematically as possible the argument runs: We wish to obtain the ratio of the activities of two solutions, one containing NaF and the other containing none. The rate of hydrolysis diminishes as the reaction proceeds, but we can represent the average activity during the production of the first 10 cc. of acid by the reciprocal of the time taken to produce that acidity. *Considering the form of the curves*, this average activity will, with sufficient accuracy for our present purposes, be also the actual activity at the point where 5 cc. of acid has been developed.

therefore  $\frac{60}{40} = 1.5$ . The concentration of the *free* sodium fluoride cannot be obtained directly, but it will be taken as equal to the total sodium fluoride present. This is, of course, not absolutely true; but we shall see in Table IV that very little of the total sodium fluoride present is combined with the enzyme, especially in weak enzyme mixtures. No appreciable error is therefore involved.

The headings in the following table are all self-explanatory. The data are derived from Table I.

TABLE II.

5 cc. enzyme solution B in a total volume of 280 cc.

NaF		TIME TO REACH ACIDITY "10"	CONC. OF ENZYME PERCENTAGES		Free Ens. NaF. Ens.	$k \times 10^5$
Mgm. per liter	Normality		Free	NaF. Ens.		
		minutes				
0.00000	0.000	53.7	100.0	0.0		
0.00893	$0.213 \times 10^{-6}$	67.8	79.2	20.8	3.81	0.812
0.0268	$0.638 \times 10^{-6}$	92.8	57.9	42.1	1.38	0.880
0.0893	$2.13 \times 10^{-6}$	190.0	28.3	71.7	0.395	0.841
0.268	$6.38 \times 10^{-6}$	450.0	11.94	88.06	0.136	0.868

\* Acidity "10" = 10 cc.  $\frac{N}{10}$  acid in 50 cc. mixture.

As an additional example a similar experiment with a crude 10 per cent extract is included. Only the final results are given.  $k$  is different in the two tables. This is due in part to slightly

TABLE III.

5 cc. enzyme solution used to 250 cc. ethyl butyrate solution.

NaF		TIME TO REACH ACIDITY "5"	CONC. OF ENZYME PERCENTAGES		Free Ens. NaF. Ens.	$k \times 10^5$
Mgm. per liter	Normality		Free Ens.	NaF. Ens.		
		minutes				
0.0008	0.000	47.9	100	0		
0.0098	$0.233 \times 10^{-6}$	53.9	89	11	8.09	1.88
0.0195	$0.464 \times 10^{-6}$	58.5	82	18	4.56	2.12
0.0481	$1.15 \times 10^{-6}$	77.1	62	38	1.64	1.87
0.0943	$2.25 \times 10^{-6}$	105.4	46	54	0.852	1.92
0.189	$4.50 \times 10^{-6}$	158.3	30	70	0.429	1.93

different conditions in the two experiments, but mainly to the fact that the enzyme solutions used were entirely different. Other experiments, performed with purified enzyme solutions, gave constants nearly equal to the ones in Table II. In every series, moreover, the values of  $k$  are constant within the limits of error of the experiment, so that the data agree satisfactorily with equation [3]. No other values for  $m$ ,  $n$  and  $p$  in equations [1] and [2] are so consistent with the observations.

If the equations as given are true the ratio<sup>6</sup>  $\frac{\text{NaF. Enz.}}{\text{Free Enz.}}$  is proportional to the free sodium fluoride. If a large amount of enzyme is used we should expect so much of the sodium fluoride to be combined that the concentration of the free sodium fluoride would be appreciably diminished. In this event, a given concentration of sodium fluoride would have less inhibiting effect in strong enzyme solutions than in weak ones. On testing this view it was found that the inhibition was apparently less in very strong solutions, but the difference was so slight as to be within the limits of experimental error. Unfortunately no great quantity of uniform purified enzyme remained for experiments in duplicate, and as it was quite evident that the enzyme was far from pure, it did not seem advisable to repeat the experiment until a much purer enzyme could be obtained. The experiment did, however, show that very little sodium fluoride was bound even in enzyme solutions of considerable strength (five times the concentration in Table II), so that the assumption made in that experiment, that the free sodium fluoride was very nearly equal to the total sodium fluoride, is justified.

The following table gives the data on which the preceding conclusion is based. Only the last column requires any explanation. This is obtained as follows: The top figures of the first five columns are obtained by extrapolation, and thus a series of figures is obtained in the fifth column giving an irregularly descending series. The total amount of sodium fluoride present is 0.030 mg. per liter, and, starting from this figure, the last column gives a regularly descending series almost directly proportional to the figures in the next to the last column.

<sup>6</sup> Note inversion of this ratio. This is done for convenience of presentation.

TABLE IV.

20 per cent purified enzyme B.  
Total volume 280 cc.

$0.714 \times 10^{-6} \text{ N.}$   
0.030 mgm. per liter.

CONCENTRATION OF ENZYME ABSOLUTE AMOUNTS		CONC. OF ENZYME PERCENTAGES		NaF. Ens. Free Ens.	CALCULATED* CONCENTRATION OF FREE NaF. (MGM. PER L.)
cc. sol. B in 280 cc.	Mgm. dried sub- stance per liter reaction mixture	Free Ens.	NaF. Ens.		
Amounts ap- proaching zero	Amounts ap- proaching zero	(52.6)	(47.4)	(0.90)	0.030 (total amount present)
5	5.7	53.	47.	0.89	0.030
10	11.4	52.	48.	0.93	0.029
25	28.6	55.	45.	0.82	0.027

\* The last column gives a uniform series, although the figures in the next to the last column do not diminish regularly.

The experiment was successful in its primary purpose; *i.e.*, it showed that in mixtures of low enzyme concentration almost all the sodium fluoride was free, but failed in its secondary purpose; *i.e.*, it did not show how much sodium fluoride was combined with a given amount of enzyme.

One of the most important points about this reaction is that it is reversible. Loevenhart and Peirce<sup>7</sup> mixed esterase and sodium fluoride and dialyzed the mixture. After dialysis the solution had regained its original activity.

The experiment was conclusive evidence for dissociation of the inactive compound, provided an inactive compound was formed under those conditions (*i.e.*, mixture of the enzyme with sodium fluoride). It is, however, possible, and indeed probable, that the presence of ethyl butyrate or alcohol or butyric acid or even two or three of these substances is necessary for the formation of the inactive compound.<sup>8</sup> The evidence for the exact nature of this inactive compound will be presented in a succeeding paper, but the question does not concern us here. The reversibility of its formation is, however, easily demonstrated.

For instance, in a 250 cc. mixture containing 153.4 cc.  $\frac{N}{10}$  ethyl butyrate, 10 cc. of enzyme and 1:6,000,000 sodium fluoride the action proceeded as if only 27.6 per cent of the enzyme present

<sup>7</sup> This *Journal*, ii, p. 406, 1907.

<sup>8</sup> For comment on these points, see Conclusion 7 at the end of this paper.

were acting. At a given time (fixed by a preliminary experiment) 5 cc.  $\frac{N}{10}$  butyric acid had been produced per 50 cc., so that 50 cc. of the mixture then contained 5 cc.  $\frac{N}{10}$  butyric acid, 5 cc.  $\frac{N}{10}$  alcohol and 25.68 cc.  $\frac{N}{10}$  ethyl butyrate. Fifty cc. of this solution were now added to 200 cc. of a mixture containing the same amount of ethyl butyrate, alcohol and butyric acid, but free from enzyme and sodium fluoride. In so doing, the enzyme and sodium fluoride were diluted five times, leaving all other factors unchanged. Two possibilities were now open for the further course of the reaction. In the first place, it might have proceeded one-fifth as fast as it did before dilution (where only 27.6 per cent of the enzyme was acting) or it might have produced acid at the same rate as a solution originally made up with 2 cc. enzyme in 250 cc. containing sodium fluoride 1:30,000,000. A control solution made up in this way worked as if about 59 per cent of the enzyme were acting, and corresponded to what was actually observed after dilution. We had, therefore, before dilution 27.6 per cent of the enzyme acting and 72.4 per cent combined with the sodium fluoride, whereas after dilution only about 41 per cent of the enzyme was present in the inactive form. The difference was great enough to be unmistakable, and gave good evidence for the fact that the reaction is reversible, whatever the nature of the inactive compound.

The data in the following experiment were obtained in the usual way, with two exceptions. First: the 50 cc. of solution to be titrated were run into 25 cc. of neutralized 80 per cent alcohol. This stopped the action more effectively than strong sodium fluoride. Second: 25 cc. instead of 50 cc. were in several instances used for a titration on account of lack of material. This accounts to a certain degree for divergence of the controls, as the titration errors must be multiplied by two.

A partial discussion of the results in the following table has just been given and the full data will now be presented.

TABLE V A.

200 cc. Ethyl butyrate solution (50 cc. = 38.35 cc.  $\frac{N}{10}$  solution).  
 10 cc. Enzyme solution C.  
 25 cc. Sodium fluoride 1:600,000.  
 15 cc. Water.

A (OBSERVED)		A (AVERAGE)		$\frac{A^*}{S}$ (CALCULATED)	
s (cc. $\frac{N}{10}$ acid)	t (min.)	s	t	S <sub>t</sub>	S <sub>t</sub> -118.7
2.01	13.08	2.11	13.94		
2.21	14.80				
3.68	29.70	3.74	30.29		
3.80	30.88				
		(4.88)	(43.7)	218.5	99.8
		(5.00)	(45.2)		
5.32	49.12	5.41	50.48	252.4	133.7
5.49	50.83				
8.85	98.43	8.94	100.01	500.0	381.3
9.03	100.58				
		(10.00)	(114.7)	573.5	454.8
10.34	120.08	10.43	120.78	603.9	485.2
10.52	121.67				

\* For explanation of columns 5 and 6, see description of Figure 2.

A second similar solution was made up (also in duplicate), and at the end of approximately 44 minutes, 50 cc. of it were added to the following solution:

134 cc. Ethyl butyrate solution  
 20 cc.  $\frac{N}{20}$  Butyric acid.  
 5 cc.  $\frac{N}{5}$  Alcohol.  
 41 cc. Water.

The first titration was made within 45 seconds of mixing and the time taken as 0 at this point.



TABLE V B.

OBSERVED		AVERAGE	
$x$	$t$	$x$	$t$
4.88	0.00	4.88	0.00
4.87	0.00		
5.39	14.62	5.44	14.85
5.49	15.08		
6.42	41.37	6.52	43.59
6.61	45.80		
7.36	74.75	7.50	75.65
7.64	76.55		
8.84	118.72	8.87	119.20
8.90	119.68		
10.42	163.80	10.28	164.63
10.14	165.45		

The two following experiments were also necessary:

TABLES V c AND V d.

C

200 cc. Ethyl butyrate solution.  
 10 cc. Enzyme solution C.  
 0 cc. Sodium fluoride.  
 40 cc. Water

D

500 cc. Ethyl butyrate solution.  
 5 cc. Enzyme solution C.  
 12.5 cc. Sodium fluoride 1:600,000.  
 107.5 cc. Water.

OBSERVED		AVERAGE		OBSERVED		AVERAGE	
$x$	$t$	$x$	$t$	$x$	$t$	$x$	$t$
4.79	12.25	4.91	12.76	2.50	42.28	2.46	41.41
5.03	13.27			2.41	41.53		
7.55	21.47	7.57	21.87	3.93	73.62	3.87	73.31
7.58	22.27			3.80	73.00		
						(4.88)	(99.8)
9.50	29.07	9.42	29.43	5.21	106.87	5.13	106.73
9.34	29.78			5.04	106.58		
		(10.00)	(31.6)				
10.92	35.20	10.69	34.70	6.28	135.47	6.17	135.61
10.45	34.21			6.05	135.75		
				7.53	175.58	7.39	175.29
				7.24	175.00		
				8.92	222.03	8.75	222.20
				8.57	222.37		
						(10.00)	(267.7)
				10.41	276.28	10.24	276.33
				10.06	276.38		

The results are also expressed graphically in the following diagram. The letters of the curves refer to the preceding table.

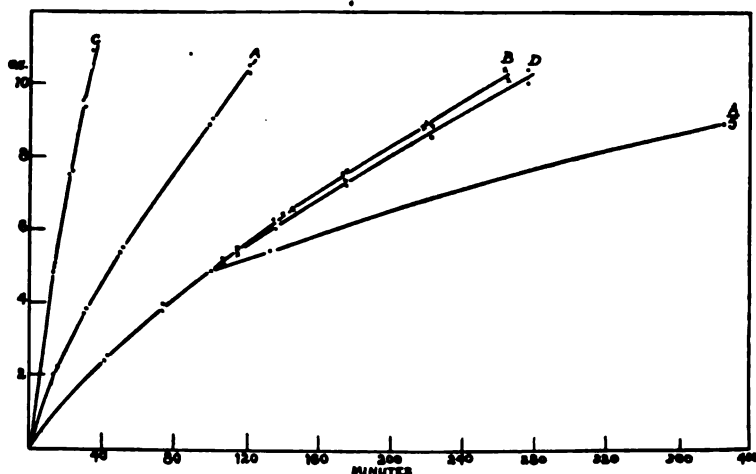


FIG. 2. TO ILLUSTRATE TABLE V.

$\frac{A}{5}$  represents a reaction going one-fifth as fast as A. A, C and D start from the origin. On the curve D, 4.88 cc. acid were produced in 99.8 minutes; but B, as observed, begins at  $x = 4.88$  and  $t = 0$ . To make the points  $x = 4.88$  on the two curves coincide, 99.8 is added to the values of  $t$  in plotting the curve B. In plotting  $\frac{A}{5}$  the values of  $t$ , for curve A, are multiplied by 5. For  $x = 4.88$ ,  $t = 218.5$ . In order to make the point  $x = 4.88$  on this curve coincide with the corresponding points on B and D, 118.7 is subtracted from the values of  $t$ .

In spite of the apparent complexity of this experiment, the point that it makes is very simple. It shows that a given mixture of enzyme, sodium fluoride, ethyl butyrate, alcohol and butyric acid, if diluted five times, with the proper mixture of ethyl butyrate, alcohol and butyric acid, is *more than one-fifth as active* as it was before dilution. Since an enzyme solution that contained no fluoride would have been only one-fifth as active, the additional activity must have come from the partial dissociation of some sort of inactive compound present in the solution. This reversibility of the formation of the inactive compound absolutely excludes destruction of the enzyme by the sodium fluoride. In

addition, the fact that curves B and D so nearly coincide, shows that the point of equilibrium demanded by equation [3] is reached almost instantly from both directions.

#### CONCLUSIONS.

1. Sodium fluoride forms a compound with the esterase from pig's liver. This compound has little, if any, hydrolytic action on ethyl butyrate.

2. The formation of this compound is reversible.

3. When the concentration of the sodium fluoride is varied from 0.00893 mgm. per liter to 0.268 mgm. per liter, the inhibition increases from 20.8 per cent to 88.06 per cent.

4. Although theoretically we should expect a given amount of sodium fluoride to have less inhibiting effect in mixtures containing a large amount of enzyme, than in weaker enzyme mixtures, the difference actually found was very slight. This indicates that in the weaker mixtures, at least, very little of the total sodium fluoride present enters into the formation of the inactive compound.

5. The following equation, based on the supposition that one molecule of the inactive compound contains one molecule of enzyme and one molecule of sodium fluoride, agrees with the observations:

$$\text{Conc. free Enzyme} \times \text{Conc. free NaF} = k \text{ Conc. (NaF. Enz.)}$$

6. The observations will not agree with an equation based on any other supposition as to the number of molecules of sodium fluoride or enzyme entering into the formation of the inactive compound. For this reason it is justifiable to conclude for the present that one molecule of the inactive compound contains only one molecule of enzyme and one molecule of sodium fluoride.

7. It is possible that ethyl butyrate, alcohol or butyric acid are also constituents of the inactive compound. This does not affect the argument in any way. It is merely necessary to consider that the "free enzyme" of the above equation represents all that enzyme which is not contained in the inactive fluoride compound. According to a preceding paper,<sup>9</sup> a large part of this

<sup>9</sup> *Journ. Amer. Chem. Soc.*, xxxi, p. 1528, 1910.

"free enzyme" is present in the form of a compound with ethyl butyrate, so that only a part of the so-called "free enzyme" is actually free. Whether the sodium fluoride combines with some compound of the enzyme or with the enzyme actually free is immaterial, provided the experiments are so arranged that the concentration of the substance with which the sodium fluoride combines is proportional to some quantity that we know. This is done by never comparing any two solutions unless the concentrations of the ethyl butyrate, alcohol, butyric acid and hydrogen ion are the same at the stage of the reaction where the two solutions are compared. Under such conditions the concentrations of all enzyme compounds, except the fluoride compound, are proportional<sup>10</sup> to the "free enzyme" of the equation, so that the mathematical treatment is justified.

<sup>10</sup> This statement must be modified if any enzyme compound that contains two molecules of enzyme is present in large amounts. Practically, there is no evidence that such compounds occur, so that for a preliminary investigation such as the present, the possibility of their existence may be neglected.

## THE RATE OF ELIMINATION OF NITROGEN AS INFLUENCED BY DIET FACTORS.<sup>1</sup>

### I. THE INFLUENCE OF THE TEXTURE OF THE DIET.

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#### INTRODUCTION.

The changing view as to the extent of digestion before absorption and the probability that proteins are split to amino-acids has raised the question whether all amino-acids are utilized alike. Are not some more resistant in metabolism than others? Does not deamination take place with greater difficulty in some cases? If it does, one can easily conceive how different may be the behavior of the various proteins in nutrition. Bearing in mind the well-known fact that proteins vary widely in chemical composition, it is evident that the products of absorption after the ingestion of one protein may be much unlike those when another is fed. Thus, if the assumption that amino-acids are of variable resistance is correct, we may have an entirely different metabolic picture in the two cases.

A study of the rate of elimination of nitrogen<sup>2</sup> in the urine suggests itself as a means of ascertaining whether or not the amino-acids behave alike in metabolism. It is obvious that any variation in the ease of deamination of the amino-acids may lead to a

<sup>1</sup> The experimental data embodied in the papers of this series are taken from the dissertation submitted by Robert C. Lewis for the degree of Doctor of Philosophy, Yale University, 1912.

<sup>2</sup> For a review of the literature on the rate of elimination of nitrogen see: Graffenberger: *Zeitschr. f. Biol.*, xxviii, p. 318, 1891; Hawk: *Amer. Journ. of Physiol.*, x, p. 115, 1903; Stauber: *Biochem. Zeitschr.*, xxv, p. 187, 1910; Wolf: *ibid.*, xl, p. 193, 1912.

change in the nitrogen-output curve after the ingestion of different amino-acids or of proteins of unlike composition. Other points, however, must be taken into consideration. Will a change in the rate of elimination of nitrogen necessarily be due to a difference in the metabolic behavior of the amino-acids? Certainly several other factors may play a part in this connection. Variations in the rate of the different processes of alimentation—gastric digestion, discharge of the food residues from the stomach and their passage along the digestive tract, pancreatic and intestinal digestion, absorption—may have a decided influence on the rapidity with which nitrogen leaves the body after a protein meal. Furthermore, metabolic processes distinct from deaminization, such as the behavior of the non-nitrogenous foodstuffs in influencing protein metabolism, are not without significance in this connection. All these factors must be considered as having a bearing in a study of the rate of elimination of nitrogen.

It seems quite probable that the lack of concordance in the nitrogen-output curves found by previous investigators may be due to a variation in certain incidental factors of the diet, such as the form in which the protein was taken, the amount of carbohydrate and fat ingested along with the protein, the water intake with the meal, and finally the proportion of indigestible material. Since the initiation of these investigations Benedickt and Roth<sup>3</sup> have suggested comparable explanations for the discrepancies in the results of earlier workers.

That indigestible materials have an influence on alimentary processes is a familiar fact. Hedblom and Cannon<sup>4</sup> have observed that coarse branny foods in the diet cause a more rapid discharge of the stomach contents. Recently Mendel and Fine<sup>5</sup> have shown that indigestible substances added to the daily meal even in small quantities cause a poorer utilization of protein. It seems quite probable, then, that the rate of elimination of nitrogen in the urine may be affected by the texture of the diet.

<sup>3</sup> Benedickt and Roth: *Zeitschr. f. klin. Med.*, lxxiv, p. 74, 1911.

<sup>4</sup> Hedblom and Cannon: *Amer. Journ. Med. Sci.*, cxxxviii, p. 1, 1909.

<sup>5</sup> Mendel and Fine: *this Journal*, xi, p. 5, 1912.

## METHODS.

The method employed in the present series of investigations for studying the rate of elimination of nitrogen in the urine has been to collect the urine at definite intervals after the ingestion of protein, to determine its content of nitrogen for the different periods, and to obtain from the results a curve of nitrogen output. Bitches were used as subjects of investigation, the urine being obtained by catheterization. The elimination of individuality was secured by the use of more than one animal for each type of experiment. In the present paper, however, it will be necessary to limit ourselves to the report of a single experiment illustrating each point. The dogs used are designated by a specific letter in the number of the experiment.

While a series of experiments was in progress the animal received each morning in a single meal a definite ration—the "Standard Diet." On experimental days the meal differed from this "Standard Diet" either by having something added to it or by having one (or more) of its constituents replaced. The same amount of nitrogen was always given, however; and in the replacement of non-nitrogenous constituents isodynamic quantities of some other foodstuff were substituted. Preceding an experiment there was always one day with the "Standard Diet" and generally there were two or three. Three of these preliminary days were introduced at the beginning of each series so that the animal might have plenty of time for adjustment to the new régime. Thus day after day throughout the whole series the food contained the same amount of nitrogen and, except in a few cases, approximately the same calorie value.

On an experimental day the animal was catheterized in the morning and fed fifteen minutes after the beginning of catheterization. To avoid possible secretory disturbances, the temperature of the well mixed food was always the same—20°C.—at the time of ingestion. Usually the animal ate the entire meal greedily. At times, however, the food had to be forced. In all cases feeding was complete in ten minutes or less. Collections of urine were made three, six, nine, twelve, fifteen and twenty-four hours after the beginning of the experiment. In some experiments a control specimen of urine was collected for an hourly period before the

commencement of the experiment; but in the majority of cases a collection for the twenty-fifth hour was made. Catheterization was planned so as to take exactly ten minutes, the expiration of that time being awaited where necessary, before the completion of the final washing. Nitrogen was determined by the Kjeldahl-Gunning method.

The "Standard Diet" was, of course, arbitrarily chosen. The only requirements in selecting such a ration were that it must furnish at least the minimal protein requisite and sufficient calories for the needs of the body. It seemed desirable, however, to use more than the minimal protein requirement in order to have a liberal output of nitrogen in the urine. The ration adopted consisted of meat,<sup>6</sup> lard, sucrose, bone ash<sup>7</sup> (5 grams), and water, with the addition in some cases of NaCl (2 grams). The water was calculated on a basis of the dry constituents, three times as much water being added as there was dry material. In other words the water was present in the whole ration in about the same proportion as it is found in meat. The sugar and lard were given in quantities approximately isodynamic to each other. The meal always contained 0.6 gram of nitrogen per kilo of body weight and furnished about 70 calories per kilo. The exact calorific value of the diets is not known because the fat content of the meat was not determined. By employing a fixed "Standard Diet" which was easy to duplicate, the rates of elimination of nitrogen under the various experimental conditions with the same and different animals were readily comparable.

Inasmuch as the addition of indigestible materials to the "Standard Diet" suggests itself as a means of studying the influence of the texture of the diet on the character of the nitrogen-output curve, mineral oil, vaseline, paraffin, filter paper; ground cork, agar-agar, bone ash, and sand were added in various experiments. There is little question that these materials pass through the intestine chemically unchanged. Bone ash may possibly be dissolved to some extent in the hydrochloric acid of the gastric juice. With respect to the mineral oil Bradley and Gasser<sup>8</sup> have reported

<sup>6</sup> Preserved frozen, according to the method of Gies.

<sup>7</sup> For the use of bone ash see Steele and Gies, *Amer. Journ. of Physiol.*, xx, p. 343, 1907.

<sup>8</sup> Bradley and Gasser: Proceedings of the American Society of Biological Chemists, December 1911, this *Journal*, xi, p. xx, 1912.



that an emulsified mixture of olive and petroleum oils fed by sound to a dog leads to absorption of both fat and hydrocarbon—a result not in accord with the later experience of Bloor.<sup>9</sup> The indigestibility of the other materials used is well established.

An amount of water equivalent to three times the weight of the superimposed substance was also added with two-fold purpose in the cases of the water-absorbing materials: filter paper, cork, and agar-agar. In the first place, without this extra water the added material could not have been soaked up and well mixed with the food. Secondly, the amount of water carried out through the bowel when these substances were used was relatively very great; and, in order to insure against a large loss of water from the tissues, it was necessary to give an added amount of water with the meal.

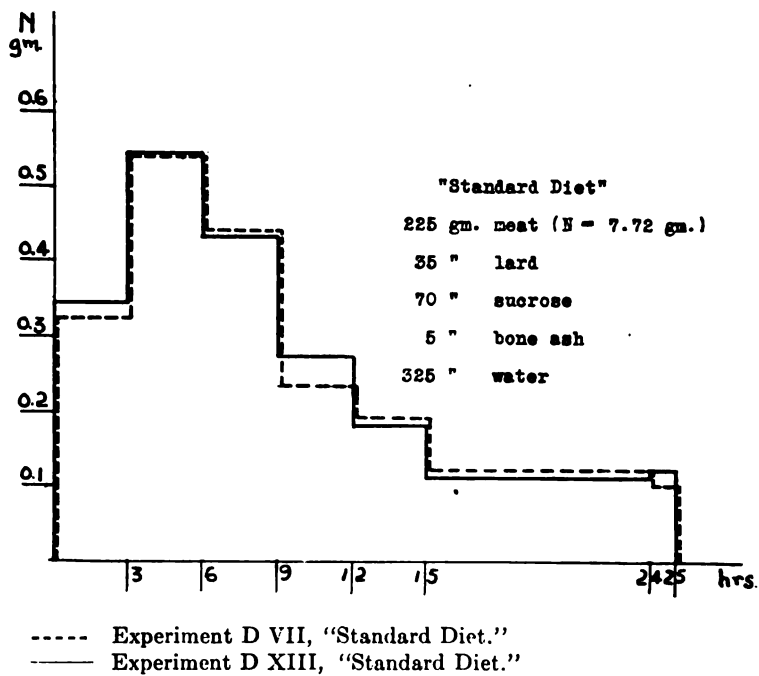
#### CONTROL EXPERIMENTS WITH THE "STANDARD DIET."

Before attempting to determine the relation of the different diet factors to the rate of nitrogen elimination in the urine, it was necessary to ascertain the nature of the nitrogen-output curve after the ingestion of the "Standard Diet" and to see whether a characteristic curve always followed. In the text each type of experiment is illustrated by a curve, plotted from the data obtained. Curve I, a typical graphic illustration,<sup>10</sup> shows the agreement of the nitrogen-output curves of two experiments with the "Standard Diet." The abscissae represent equal increments of time; the ordinates, grams of nitrogen. Thus at a glance the average *hourly* rate of elimination of nitrogen for a single period is shown by the value of the ordinate. It is readily seen that after the ingestion of the "Standard Diet" there is a rise in the nitrogen output during the first period, reaching a maximum in the second three hours, followed by a fall to the initial level early the next morning. In the present work it has always been possible with the same animal to get "standard" experiments which agree within reasonably close limits (Curve I). Furthermore "standard" curves of duplicate character have been obtained repeatedly with different animals.

<sup>9</sup> Bloor: *This Journal*, xv, p. 105, 1913.

<sup>10</sup> All curves show the rate of nitrogen output in two experiments, a "standard" experiment (broken line) being plotted for purposes of comparison.

CURVE I. To illustrate the agreement of duplicate experiments after the ingestion of the "Standard Diet."

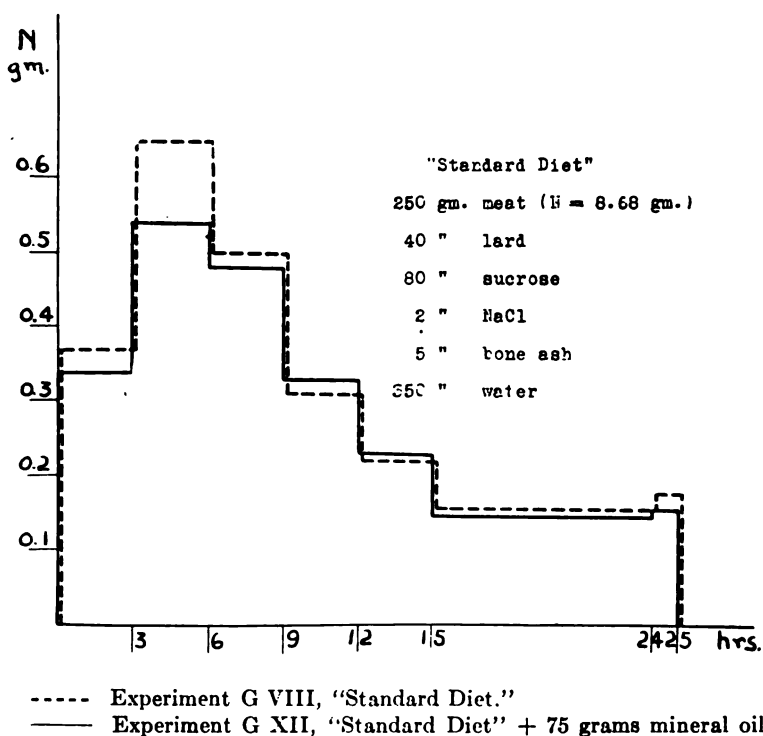


## EXPERIMENTS WITH INDIGESTIBLE MATERIALS.

*Mineral oil*<sup>11</sup> (Curve II).

When mineral oil was added to the diet the nitrogen output in the second period was notably less than in the corresponding period after the ingestion of the "Standard Diet" alone. Evidently mineral oil causes a slower rate of elimination of nitrogen.

CURVE II. To illustrate the effect of an addition of *mineral oil* to the "Standard Diet" on the rate of elimination of nitrogen.



<sup>11</sup> A colorless, purified product sold under the trade name of "Alboline."

The effect of vaseline on the curve of nitrogen elimination is similar to, but more marked than that of mineral oil. The nitrogen output in each of the first three periods is smaller than in the "standard" experiment; afterwards the two curves are almost identical. There is a delay in the excretion of nitrogen.

**"Standard Diet"**  
 225 gm. meat (N = 7.27 gm.)  
 35 " lard  
 70 " sucrose  
 5 " bone ash  
 325 " water

Time (hrs.)	Experiment D IV (N gm.)	Experiment D VI (N gm.)
0-3	0.33	0.33
3-6	0.58	0.48
6-9	0.40	0.32
9-12	0.22	0.22
12-15	0.14	0.14
15-24	0.09	0.09
24-25	0.00	0.00

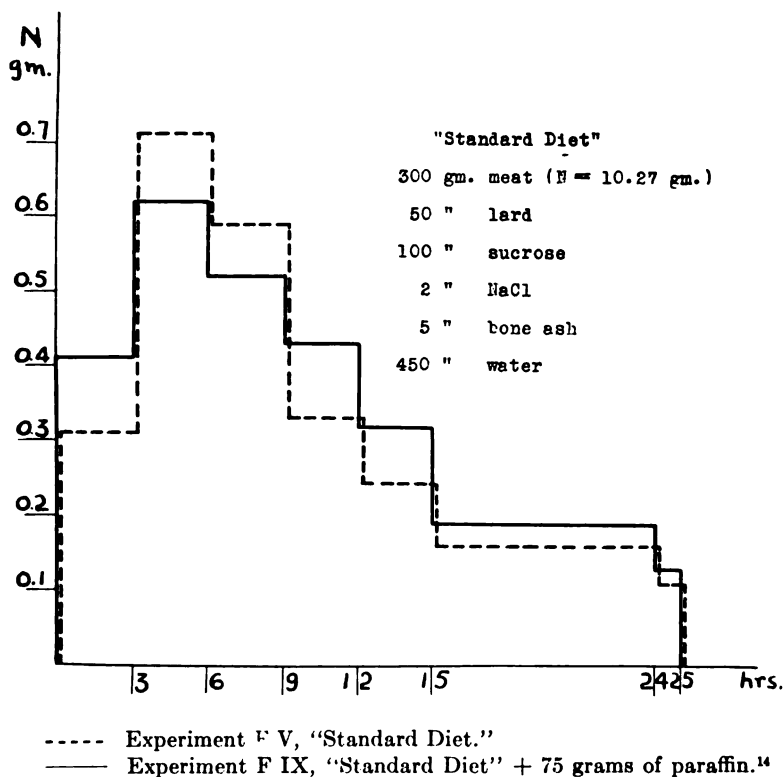
----- Experiment D IV, "Standard Diet."  
 — Experiment D VI, "Standard Diet" + 75 grams vaseline.

<sup>12</sup> Yellow petroleum jelly (M.P.=38°C.).

*Paraffin*<sup>13</sup> (Curve IV).

The experiment with paraffin shows a much more decided flattening of the nitrogen-output curve, *i.e.*, a preliminary delay in excretion of nitrogen, than was the case with either of the softer petroleum products. The rate of elimination of nitrogen is not only lower in the earlier periods than in the "standard" experiment, but also higher during the latter part of the day.

CURVE IV. To illustrate the effect of an addition of *paraffin* to the "Standard Diet" on the rate of elimination of nitrogen.



<sup>13</sup> Fine shavings, obtained by scraping a cake of paraffin (M.P. = 51°C.) with a knife.

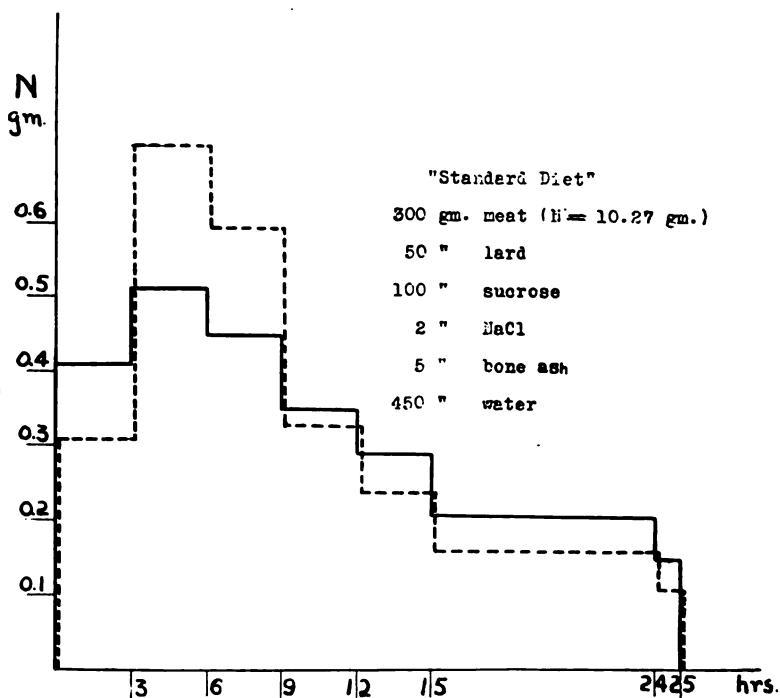
<sup>14</sup> Large quantity of paraffin feces during the night (15–24 hour) period.

## Rate of Nitrogen Elimination

*Filter Paper*<sup>15</sup> (Curve V).

The rate of elimination of nitrogen during the earlier periods after the ingestion of the "Standard Diet" plus filter paper is lower than in the "standard" experiment; during the later hours it is higher than normal. Thus, as was the case with paraffin, there results a very decided flattening of the nitrogen-output curve.

CURVE V. To illustrate the effect of an addition of *filter paper* to the "Standard Diet" on the rate of elimination of nitrogen.



----- Experiment F V, "Standard Diet."

———— Experiment F VIII, "Standard Diet" + 75 grams filter paper<sup>16</sup> and 225 grams water.

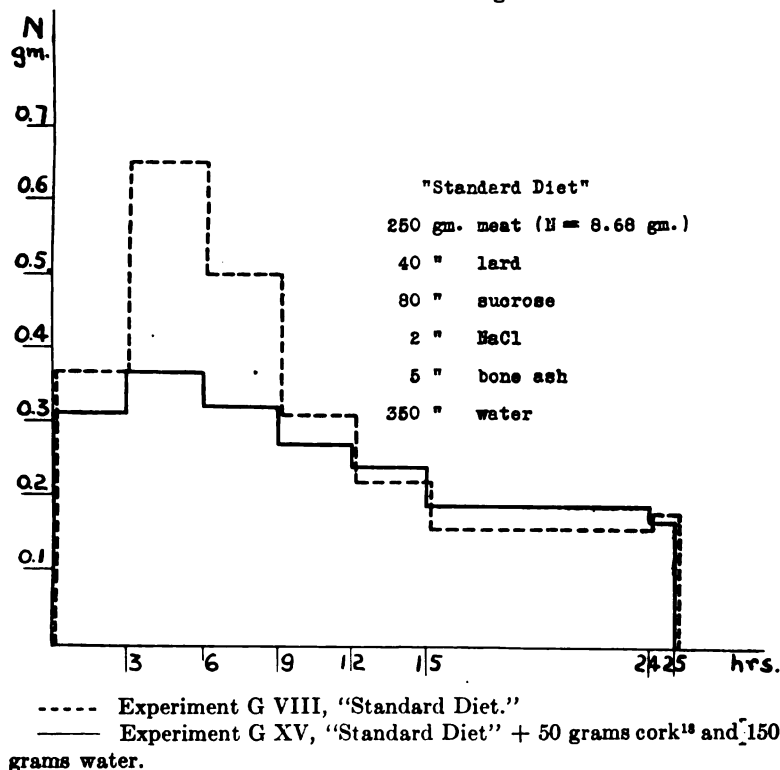
<sup>15</sup> Cut up in small pieces.

<sup>16</sup> Large quantities of paper feces during the 4th and 5th three-hour periods and during the night (nine-hour) period.

*Cork*<sup>17</sup> (Curve VI).

With cork there is likewise a much slower elimination of nitrogen than with the "Standard Diet" alone. In this case the total nitrogen output for the entire day is lower than in the "standard" experiment. This, however, is not the only cause of the sub-normal elimination of nitrogen in the *early periods* of the day; for the character of the nitrogen-output curve is radically different from the "standard"—lower during the early periods and higher during the later periods of the day.

CURVE VI. To illustrate the effect of an addition of *cork* to the "Standard Diet" on the rate of elimination of nitrogen.



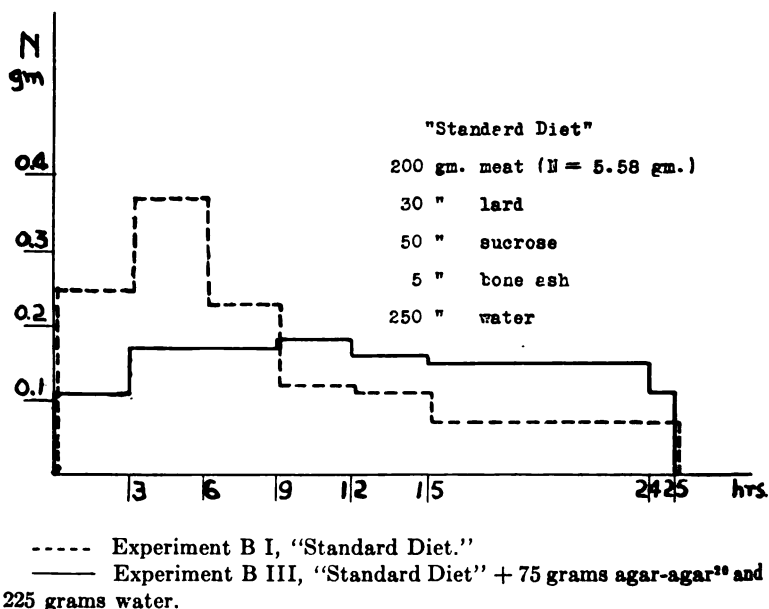
<sup>17</sup> Finely ground in a coffee mill.

<sup>18</sup> Large quantities of cork feces during the 4th and 5th three-hour periods and during the night (nine-hour) period.

*Agar-agar*<sup>19</sup> (Curve VII).

Of all the indigestible materials used the agar-agar caused the most pronounced delay in the nitrogen output. In the experiment here reported there was a rise in the second period over the value of the first three hours and then very little change for twelve hours; in other experiments there was a similar though slightly less marked effect.

CURVE VII. To illustrate the effect of an addition of *agar-agar* to the "Standard Diet" on the rate of elimination of nitrogen.



<sup>19</sup> Very finely chopped.

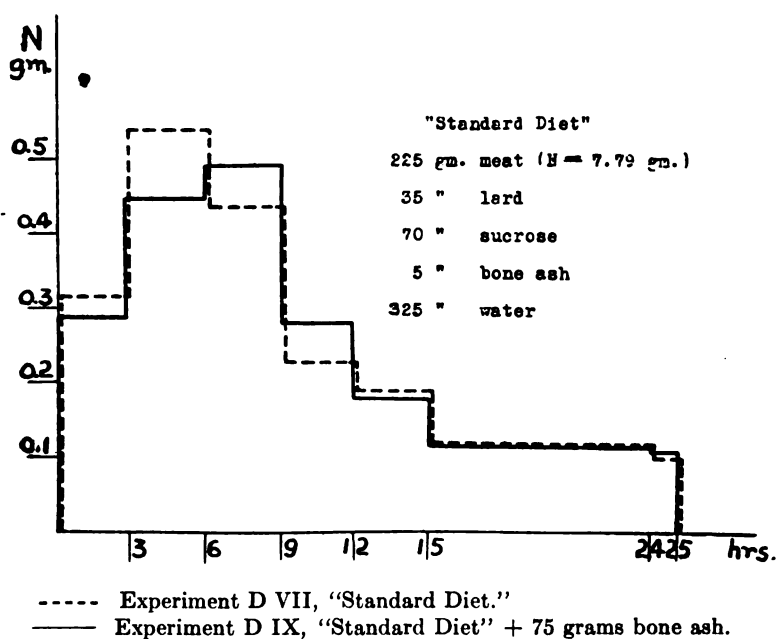
<sup>20</sup> First agar-agar feces during first three-hour period. No note kept of subsequent defecations; feces in almost every period, however.



*Bone Ash (Curve VIII).*

With the addition of bone ash to the "Standard Diet" there is a flattening of the curve, but by no means to such an extent as with any of the previously mentioned indigestible materials except the softer petroleum products. There is a delayed excretion of nitrogen in the first two periods, followed by a slight compensatory rise during the next six hours, the curve afterward running parallel to that of the "standard" experiment.

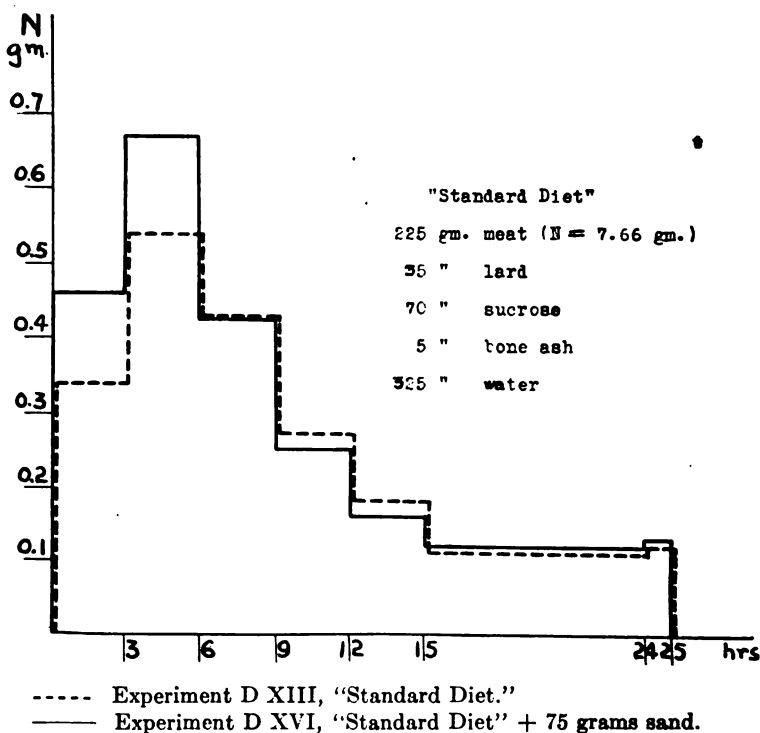
CURVE VIII. To illustrate the effect of an addition of *bone ash* to the "Standard Diet" on the rate of elimination of nitrogen.



*Sand* (Curve IX).

The effect on the nitrogen-output curve of an addition of very fine sand to the "Standard Diet" is entirely different from anything so far reported. The rate of elimination of nitrogen during the first two periods is notably *higher* than in the corresponding periods of the "standard" experiment; afterward the two curves run parallel.

CURVE IX. To illustrate the effect of an addition of *sand* to the "Standard Diet" on the rate of elimination of nitrogen.



## DISCUSSION.

The experiments with a variety of indigestible materials have shown a slower rate of elimination of nitrogen after the addition of these substances to the "Standard Diet" except in the case of sand. Obviously there has been some delay in the processes of alimentation; for, excepting differences in the amount of water absorbed,<sup>21</sup> the purely metabolic conditions are the same when an indigestible substance is included in the daily meal as when the "Standard Diet" alone is fed. The prime factor in this delay must have been a slower rate of absorption, whether induced by a retardation of the discharge of the gastric contents, a delay in digestion, an adsorption of digestive products by the indigestible material, or a loss of absorbable material by an early evacuation of the bowel. Let us consider the bearing of each of these contributory factors on the present work.

In all probability no delay in the *discharge from the stomach* occurred when indigestible materials were added to the diet. With mineral oil, vaseline, paraffin, and bone ash the passage of food onward must have been as rapid as under normal conditions; for during the first periods with these materials there was no marked decrease in the nitrogen output below that of the "standard" experiment. The early appearance of the added indigestible material in the feces following the ingestion of filter paper, cork, and agar-agar<sup>22</sup> suggests an acceleration rather than a retardation of the normal gastric discharge. This is in harmony with the report of Hedblom and Cannon<sup>23</sup> that branny foods cause a more rapid emptying of the stomach.

A delayed absorption on account of a sub-normal *rate of digestion* in the experiments with indigestible materials is quite possible.

<sup>21</sup> With several of the indigestible substances—cork, filter paper, and agar-agar—a large amount of water was excreted through the bowel. As a result the volume of urine, and hence the water absorbed, was very small. That the lack of water was not an important factor in causing a retardation of the rate of elimination of nitrogen has been shown by an experiment with agar-agar in which a very large amount of water was given. In this case there was a normal flow of urine, but the same effect was obtained as when the secretion of urine was small.

<sup>22</sup> The influence of these substances on the emptying of the bowel is indicated in the reports of the experiments.

<sup>23</sup> Hedblom and Cannon: *Amer. Journ. Med. Sci.*, cxxxviii, p. 1, 1909.

It was pointed out in the preceding paragraph that some of the indigestible materials caused a more rapid discharge from the stomach. This might very well lead to a slower rate of digestion because the preliminary gastric proteolysis was inhibited before it had advanced very far, and the intestine thus received much more complex residues than under normal conditions. This possibility of an unfavorable result from sparing the stomach at the expense of the intestine has been previously mentioned by Cohnheim<sup>24</sup> and Cannon.<sup>25</sup> It is interesting to note that it was in the cases where there was an early emptying of the stomach that the greatest delay in elimination of nitrogen, and hence in absorption, occurred. Furthermore, digestion may have been delayed because the food residues, mixed as they must have been with the indigestible materials, are rendered more or less inaccessible to the action of the digestive enzymes.

Another possible explanation for the delay in absorption when indigestible substances are added to the diet is that the *products* of digestion are *adsorbed* by the added indigestible material. It is quite conceivable that agar-agar, for example, might adsorb the soluble intestinal contents. An examination of the data shows that the indigestible materials exerted a retarding influence on the rate of absorption, as measured by the rate of elimination of nitrogen. This retardation was progressively greater in the following order—mineral oil, vaseline, bone ash, paraffin, filter paper, cork, agar-agar—corresponding with the comparative adsorptive power of the same substances. This parallelism between delayed absorption and power of adsorption seems to be more than a coincidence. Adsorption of intestinal contents by the indigestible adjuvants might cause a slower rate of absorption in two ways: in the first place, the partially digested protein residues might in part be rendered less accessible to enzyme action; secondly, a smaller proportion of the completely digested protein residues would come in contact with the intestinal wall, and for this reason absorption would be hindered.

It is unlikely that the delayed absorption in the experiments with indigestible materials is attributable to an early *emptying*

<sup>24</sup> Cohnheim: Quoted by Cannon.

<sup>25</sup> Cannon: *The Mechanical Factors of Digestion*, International Medical Monographs I (Longmans, Green and Company), 1911, p. 123.

of the bowel and a consequent loss of available nitrogen. The more than compensatory rise in the urinary nitrogen output above the normal which always occurs in the later periods with the substances causing early evacuation makes such an explanation questionable.

There remains a consideration of the results obtained with sand. The data presented emphasize an increased elimination of nitrogen in the first periods after the meal when sand is added to the "Standard Diet." Can this rise be caused by a more rapid discharge of the gastric contents? No evidence has been found in the literature to warrant such a conclusion. On the contrary the results of Hedblom and Cannon,<sup>26</sup> showing that hard irregular pieces of dried starch paste in the diet caused a slower discharge of the stomach, appear to speak against such an explanation. It is unlikely that sand has mechanically stimulated an increased secretion, the reabsorption<sup>27</sup> of which has raised the nitrogen output of these first two periods; for Pawlow<sup>28</sup> has demonstrated conclusively that the blowing of sand with force against the walls of the inactive stomach does not stimulate gastric secretion. The failure of sand to stimulate secretion is further shown by the following experiment:

During the middle of the fourth day of a fast the urine was collected for a three-hour control period. A quantity of sand (and a little water) was then given; and the urine was subsequently collected at three-hour intervals for nine hours. There was no increase in the nitrogen output of these later periods over that of the control period.<sup>29</sup>

<sup>26</sup> Hedblom and Cannon: *Amer. Journ. Med. Sci.*, cxxxviii, p. 1, 1909.

<sup>27</sup> Mosenthal (*Journ. Exp. Med.*, xiii, p. 319, 1911) has attributed to the intestinal secretion a considerable source of absorbable nitrogen. From experiments on dogs with isolated loops of intestine he estimated that the nitrogen content of the succus entericus secreted in twenty-four hours was equivalent to about 35 per cent of the nitrogen intake. Inasmuch as the feces contained nitrogen equivalent to only 10 per cent of the intake, the major part of the intestinal secretion must have been reabsorbed.

<sup>28</sup> Pawlow: *The Work of the Digestive Glands*, translated by W. H. Thompson (Charles Griffin and Company), 1902, pp. 86-90.

<sup>29</sup> In a previous experiment we had ascertained that the nitrogen-output curve during starvation varied but little from a straight line.

## SUMMARY.

The typical curve of nitrogen elimination on a selected mixed diet shows a rise in the first period, reaching a maximum in the second three hours, followed by a fall to the initial level early the next day.

With a definite diet it has always been possible to duplicate experiments on the same animal. Different animals on the same type of diet have given parallel curves.

A delay in the elimination of nitrogen is caused by the addition to the diet of such indigestible materials as mineral oil, vaseline, bone ash, paraffin, filter paper, cork, and agar-agar—substances which act in a purely mechanical as contrasted with a chemical manner. Invariably there is a subnormal rate of nitrogen output in the first periods following ingestion of the meal; with paraffin, filter paper, cork, and agar-agar this is followed by a higher rate in the later periods. The effect of the indigestible materials is progressively greater in the order in which they are given above.

A delayed absorption of the nitrogen intake is presumed responsible for the slower rate of elimination of nitrogen. As possible causes of this retardation of absorption the following have been suggested: (1) a slower rate of digestion caused by an early emptying of the stomach and a consequent early exclusion of gastric proteolysis, with the possibility of a more prolonged intestinal digestion; (2) a slower rate of digestion caused by an adsorption of partially digested protein residues by the added indigestible material, making them less readily accessible to the action of the digestive enzymes; (3) an adsorption of the final digestive products by the indigestible substance whereby their absorption from the intestine is hindered.

Sand gives an exception to the results obtained with the other indigestible materials studied, as it causes an elimination of nitrogen above the normal in the first six hours. This rise is presumably not caused by an increased secretion and subsequent reabsorption of digestive juices, for the ingestion of sand during starvation has no effect on the nitrogen-output curve.

## THE RATE OF ELIMINATION OF NITROGEN AS INFLUENCED BY DIET FACTORS.

### II. THE INFLUENCE OF CARBOHYDRATES AND FATS IN THE DIET.

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(Received for publication, August 4, 1913.)

Although the general effect of carbohydrates in the diet on the rate of elimination of nitrogen in the urine seems to be well established, no study of the comparative behavior of different carbohydrates has been attempted. In the past the method of investigation employed has been to superimpose the carbohydrate to be studied on a "standard" diet, to determine the rate of elimination of nitrogen after this augmented meal, and to ascertain the effect of the carbohydrate on the nitrogen-output curve by contrast with the rate when the "standard" diet alone was fed. The objection to this method is that on the day when carbohydrate is given the diet has a greater calorific value than the "standard" diet, so that the conditions on the two experimental days are not comparable from the standpoint of energy intake. In the present investigation<sup>1</sup> an isodynamic quantity of carbohydrate was substituted for the non-nitrogenous constituents of the "Standard Diet," and thus the calorie value of the food of all days remained the same. The following carbohydrates were chosen for study: the polysaccharides, starch and soluble starch; the disaccharide, sucrose; and the monosaccharide, dextrose.

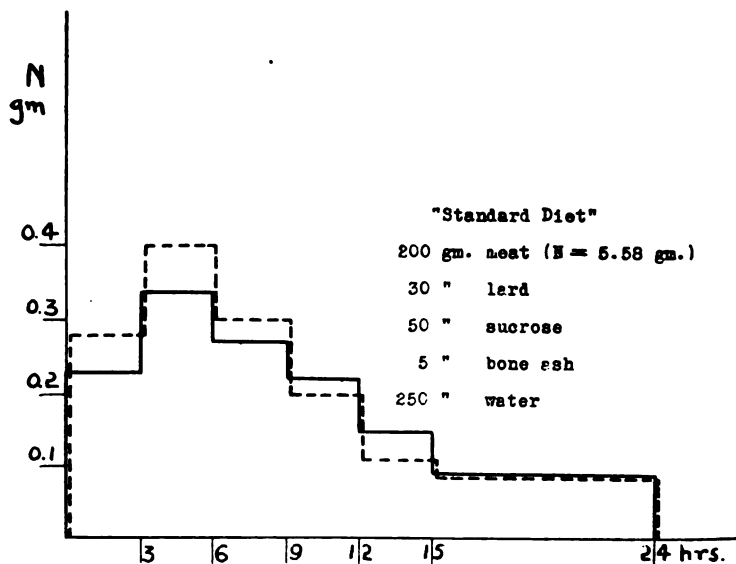
<sup>1</sup> The methods employed were those outlined in our first paper (cf. this *Journal*, xvi, p. 19, 1913).

## EXPERIMENTS WITH CARBOHYDRATES.

*Starch*<sup>2</sup> (Curve I).

When starch was substituted for the non-nitrogenous constituents of the "Standard Diet" there was a distinct delay in the elimination of nitrogen, the amount of nitrogen excreted being smaller than in the "standard" experiment in the earlier periods of the day and larger in the later periods.

CURVE I. To illustrate the effect on the rate of nitrogen elimination of substituting *starch* for the non-nitrogenous constituents of the "Standard Diet."



---- Experiment A IX, "Standard Diet."

— Experiment A XIV, lard and sucrose of "Standard Diet" replaced by starch (118 grams).

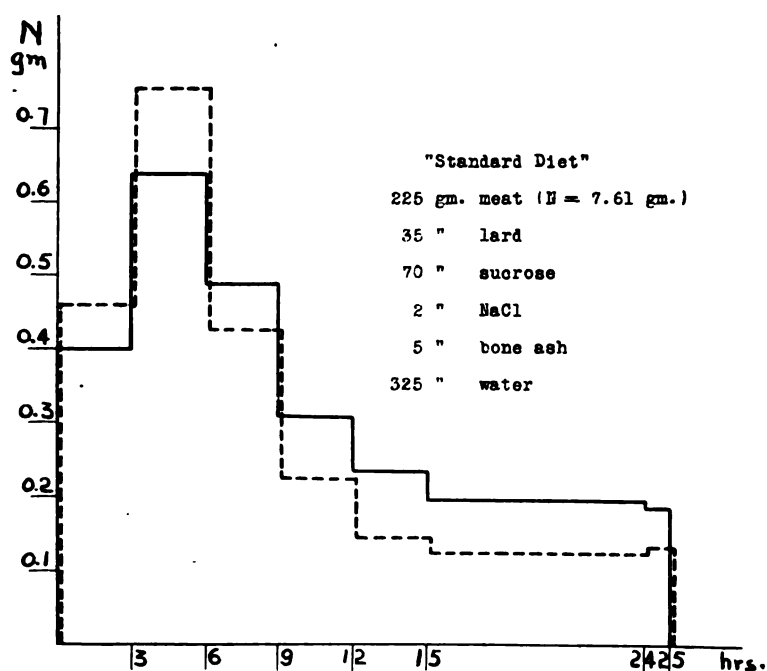
<sup>2</sup> Arrowroot starch was mixed with water and heated in an autoclave for fifteen minutes in order to rupture the starch grains.



*Soluble starch* (Curve II).

With the use of soluble starch in place of starch the retarding effect on the nitrogen excretion was even more marked.

CURVE II. To illustrate the effect on the rate of nitrogen elimination of substituting *soluble starch* for the non-nitrogenous constituents of the "Standard Diet."



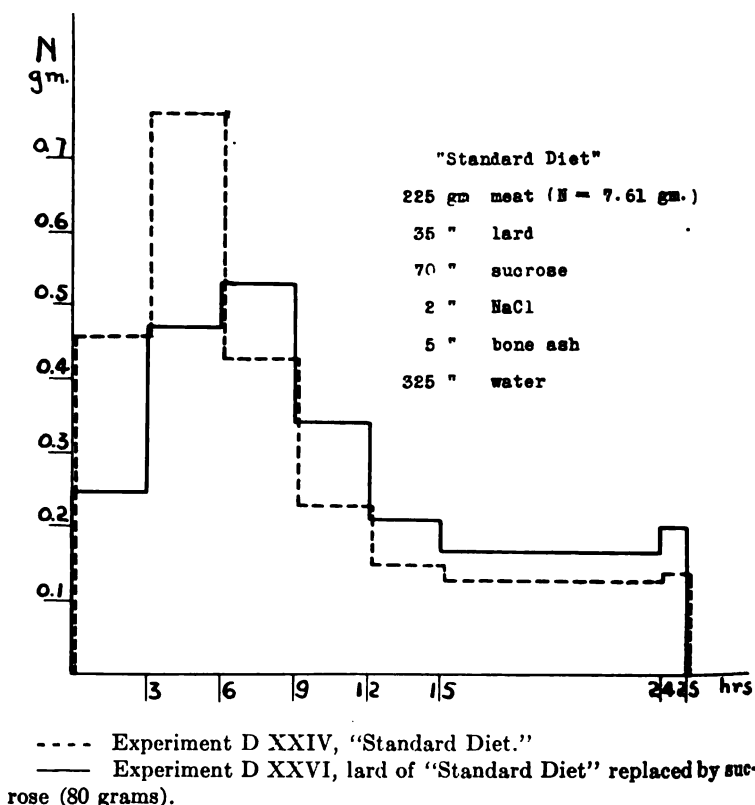
----- Experiment D XXIV, "Standard Diet."

———— Experiment D XXVII, lard and sucrose of "Standard Diet" replaced by soluble starch (150 grams).

*Sucrose* (Curve III).

When sucrose was substituted for the lard of the "Standard Diet" the maximum output of nitrogen did not occur until the third three-hour period, as contrasted with the second period in the "standard" experiment and in experiments with the carbohydrates above reported. The nitrogen output was relatively much smaller in the earlier periods of the day and much larger in the later periods than was the case with either of the polysaccharides. In other words the flattening effect of sucrose on the nitrogen-output curve was much more pronounced than that of starch or of soluble starch.

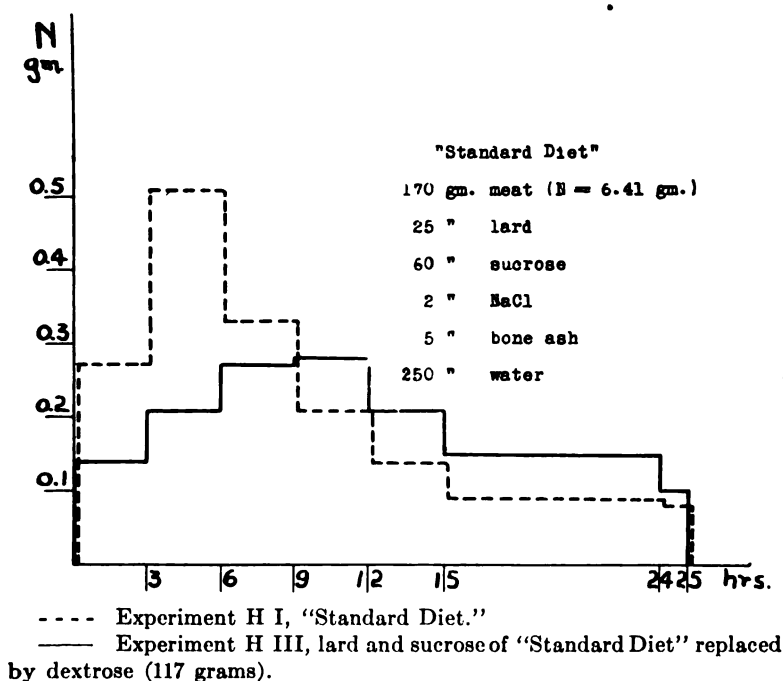
CURVE II. To illustrate the effect on the rate of nitrogen elimination of substituting *sucrose* for the lard of the "Standard Diet."



*Dextrose (Curve IV).*

With dextrose replacing the non-nitrogenous constituents of the "Standard Diet" the preliminary delay in nitrogen excretion was much greater than with any of the other carbohydrates studied, the nitrogen-output curve having a much more flattened aspect.

CURVE IV. To illustrate the effect on the rate of nitrogen elimination of substituting *dextrose* for the non-nitrogenous constituents of the "Standard Diet."



*Sucrose; Lard being present in the diet.*

In the experiments above reported the diets were fat-free and in this respect not comparable with the "Standard Diet." What effect would an increased amount of sucrose, for example, have on the rate of nitrogen elimination if lard were also present in the diet? In a further experiment the meal contained an amount of sucrose isodynamic to the non-nitrogenous constituents of the

"Standard Diet" and enough fat (94 grams of lard) to make the ratio of sugar to fat approximately the same as in the "Standard Diet." With such a procedure an increased number of calories was given, but to accomplish the desired end this was necessary. There was the same preliminary delay in the nitrogen excretion in this case with sucrose as when no fat was present in the diet.

#### DISCUSSION.

The substitution of the different carbohydrates for the non-nitrogenous constituents of the "Standard Diet" resulted in a slower rate of elimination of nitrogen, a flattening of the nitrogen-output curve. The carbohydrates studied were progressively more effective in the following order: starch, soluble starch, sucrose, and dextrose.

With the method employed in the present study with carbohydrates two important changes have been made in the diet: (a) fat has been removed; (b) an added amount of carbohydrate has been given. Which of these two changes is of paramount importance in causing the results above reported? An experiment in which fat as well as sucrose was added to the "Standard Diet" shows that the removal of the fat was not the causal factor; for here, as in the cases where sucrose replaced lard, the curve of nitrogen elimination is considerably flattened despite the presence of an abundance of fat. The added carbohydrate must have been directly responsible, then, for the slower rate of nitrogen elimination.

The results of the present investigation with carbohydrates are completely in harmony with those of previous investigators. Vogt (1906) found that the addition of rice or rice flour to a meat diet caused a slower rate of elimination of nitrogen than meat alone. Levene and Kober (1909) reported that with the addition of starch to a "standard" diet containing plasmon, cracker meal, and lard the course of nitrogen elimination did "not differ materially from that of the standard diet." A careful examination of the data presented by these authors discloses, however, a slight flattening of the nitrogen-output curve when starch was added to the diet. According to Van Slyke and White (1911) starch superimposed on a diet of fish, cracker meal, and lard caused a delayed elimination of nitrogen of about the same magnitude as in the starch experiments of the present work. Falta and Gigon (1908)

found a delayed excretion of nitrogen after the addition of either wheat flour or levulose to a meat diet, the effect being more marked in the case of the latter carbohydrate. This result with levulose agrees with that of Falta, Grote, and Staehelin (1907). Pari (1908) reported that with the addition of sucrose to a meat diet there was a retardation of the nitrogen excretion. Interesting in this connection are the experiments of Boettcher and Vogt (1909) in which subcutaneous injections of dextrose (5-10 grams) caused a flattening of the nitrogen-output curve. All of these investigators worked with dogs.<sup>3</sup> Lusk (1912) fed dextrose alone to dogs 24 hours after the last meal and found a nitrogen output lower than the fasting level during the hour following the dextrose intake. Subsequently there was a compensatory rise in the nitrogen elimination.

Concerning the manner in which carbohydrate may be responsible for a delay in nitrogen excretion several possibilities must be considered, viz: a subnormal rate of discharge of the stomach contents, a retardation of digestion, a delayed absorption, altered metabolic processes. Van Slyke and White (1911) have given no experimental proof for their conclusions that the retardation of nitrogen elimination when starch is added to the diet is caused by a delay in digestion and absorption. The experiments of Boettcher and Vogt (1909), showing a delay in absorption after subcutaneous or intravenous dextrose injections in five out of seven cases, are hardly comparable with the experiments of the present series where the carbohydrate was given *per os*. In fact, no conclusive evidence has been found in the literature to the effect that a subnormal rate of any of the alimentary processes is caused by an addition of carbohydrate to the diet. On the contrary, the report of Cannon<sup>4</sup> that a mixture of carbohydrate and protein foods leaves the stomach more rapidly than protein alone, whereas fat has a retarding action on the emptying of the stomach, makes it probable that the addition of carbohydrate to, and the removal of fat from the diet in the present experiments is, if anything, followed by a more rapid discharge of the gastric contents than in the "standard" experiment.

<sup>3</sup> Wolf (1912) studied the rate of elimination of nitrogen after the ingestion of starch by a fasting man. The results have no bearing on the experiments here reported.

<sup>4</sup> Cannon: *Amer. Journ. of Physiol.*, xii, p. 387, 1904.

There is some evidence in the literature that variations in metabolic processes are responsible for the slower rate of nitrogen elimination under the influence of carbohydrates. Falta and Gigon (1908) and Par. (1908) have attributed this delay to the protein-sparing action of carbohydrates; for after a fast, when the glycogen depots are almost depleted, the carbohydrates no longer exert a retarding action on the nitrogen-output curve. The reason for this, according to these authors, is that the carbohydrates now go to make up the depleted glycogen supply in preference to being immediately burned. Boettcher and Vogt (1909) think that a disturbance of intermediary metabolism is in part responsible for the delay in nitrogen excretion obtained after subcutaneous dextrose injections, although they offer no experimental proof for their contention. The consensus of opinion, then, seems to favor a disturbance of metabolic processes, rather than a delay in alimentation, as the causal factor in the retardation of nitrogen excretion when carbohydrate is present in the diet.

Although the experimental data obtained in this study do not warrant the adoption of a final theory as to how the carbohydrates act to retard nitrogen excretion, the writer is inclined to the belief that the protein-sparing action of carbohydrate causes this delay. When carbohydrate is present in the diet, it is digested, absorbed, and burned; while the protein residues, which are simultaneously absorbed, are temporarily spared to some extent and are only completely metabolized when carbohydrate is no longer available, a preliminary delay in nitrogen excretion thus occurring. If such a theory holds, the physiological, six-carbon sugar dextrose should be more efficient than the polysaccharide starch in causing a retardation of nitrogen excretion; for the more nearly the carbohydrate is prepared for absorption when ingested, the sooner should its sparing action come to expression, and so the greater should be the delay in nitrogen elimination. As a matter of fact the carbohydrates studied did show a progressively greater retarding effect in the order: starch, soluble starch, sucrose, dextrose. Thus the theory accounts for all the results obtained.

In conclusion reference should be made to the effect of indigestible carbohydrates in the diet on the rate of elimination of nitrogen. In the previous paper<sup>5</sup> it was shown that cellulose—filter paper

<sup>5</sup> Mendel and Lewis: *This Journal*, xvi, p. 19, 1913.

and cork—and agar-agar caused a delay in nitrogen excretion. It is obvious, however, that the explanations of the similar effect on the rate of nitrogen elimination after the ingestion of digestible and indigestible carbohydrates, respectively, are radically different.

#### SUMMARY OF RESULTS WITH CARBOHYDRATES.

Carbohydrates in the diet cause a slower rate of elimination of nitrogen after a protein meal, the various carbohydrates studied having a progressively greater effect in the following order: starch, soluble starch, sucrose, dextrose.

The experimental data do not warrant the adoption of more than a tentative theory as to the explanation of the retardation of nitrogen excretion when carbohydrates are present in the diet. It seems quite probable, however, that the protein-sparing action of carbohydrate is responsible for this delay. At any rate all the results obtained in the experiments with carbohydrates may be explained by such a theory.

#### THE RELATION OF FATS IN THE DIET TO THE RATE OF ELIMINATION OF NITROGEN.

No comparative study of fats of different texture—soft or hard—has been attempted in the few previous investigations of the influence of fat in the diet on the rate of nitrogen elimination. Most of the work in the past has been done by superimposing the fat on a “standard” diet, and determining its effect on the nitrogen-output curve. Inasmuch as this procedure is open to the objection that with the addition of fat to the diet an increased number of calories is given, a method similar to that employed in the study of carbohydrates<sup>6</sup> was adopted for fats, the non-nitrogenous constituents of the “Standard Diet” being replaced by the fat to be studied. The following fats of widely different textures were used: cotton-seed oil, lard, and “Oleo-stearin”<sup>7</sup> (M.P. = 53°C.).

<sup>6</sup> See the first part of this paper.

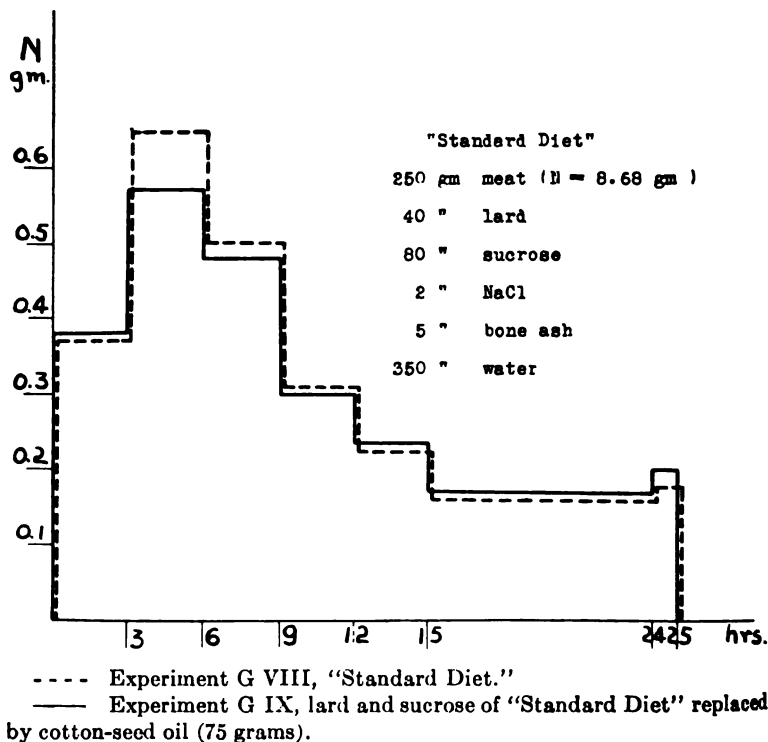
<sup>7</sup> Armour and Company kindly furnished this product.

## EXPERIMENTS WITH FATS.

*Cotton-seed oil* (Curve V).

The substitution of cotton-seed oil for the non-nitrogenous constituents of the "Standard Diet" had very little effect on the course of the nitrogen-output curve, the only variation from the "standard" occurring in the second three-hour period where the nitrogen output was decreased.

CURVE V. To illustrate the effect on the rate of nitrogen elimination of substituting *cotton-seed oil* for the non-nitrogenous constituents of the "Standard Diet."

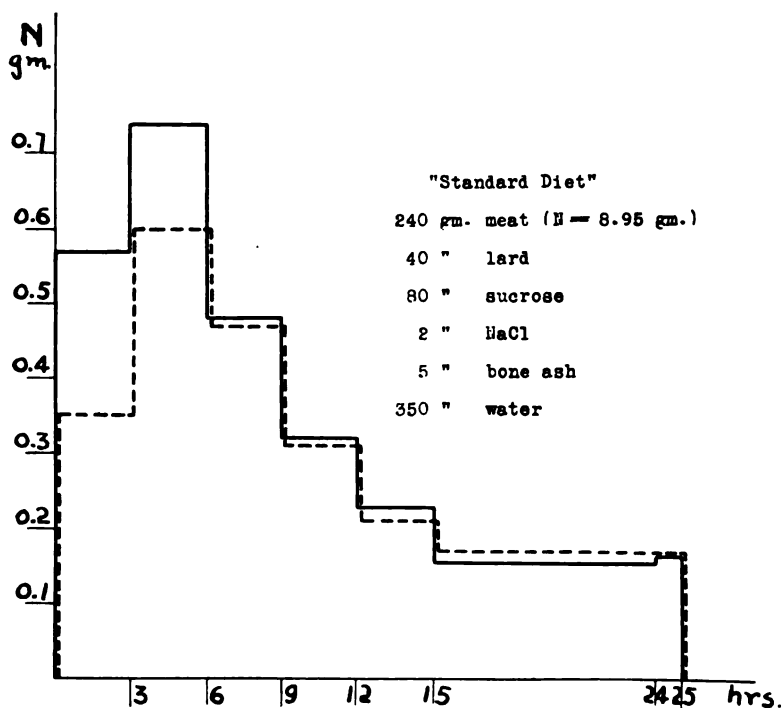




*Lard* (Curve VI).

When lard was substituted for the sucrose of the "Standard Diet," the nitrogen excretion during the first two three-hour periods was considerably larger than in the "standard" experiment; afterwards the nitrogen-output curve was identical with that after the ingestion of the "Standard Diet."

CURVE VI. To illustrate the effect on the rate of nitrogen elimination of substituting *lard* for the sucrose of the "Standard Diet."

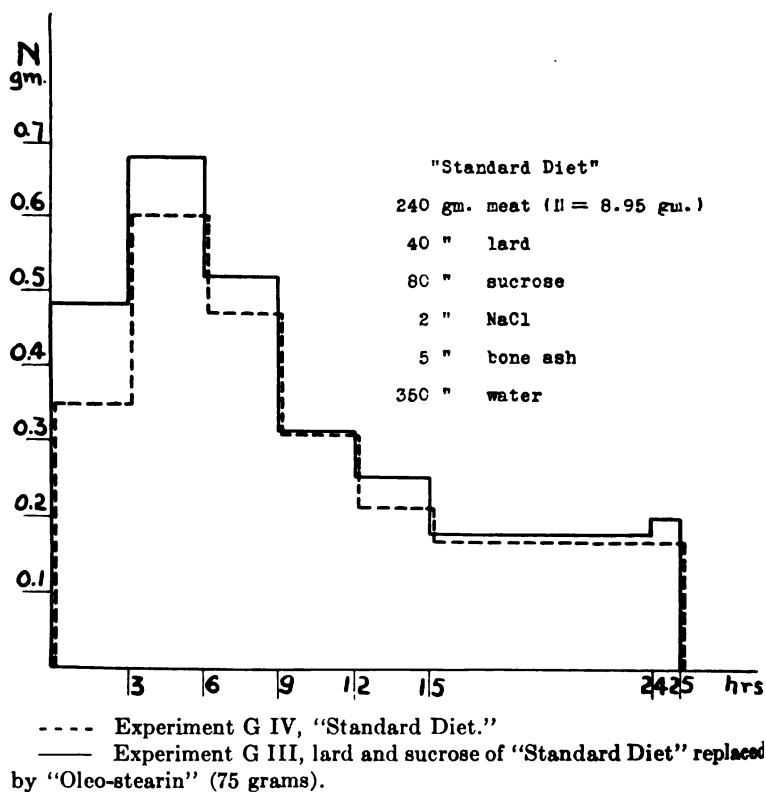


----- Experiment G IV, "Standard Diet."  
 ——— Experiment G VI, sucrose of "Standard Diet" replaced by lard (35 grams).

*"Oleo-stearin" (Substitution) (Curve VII).*

The result of *substituting* "Oleo-stearin" for the non-nitrogenous parts of the "Standard Diet" was similar to that with lard, a nitrogen output above the normal occurring in the first three three-hour periods.

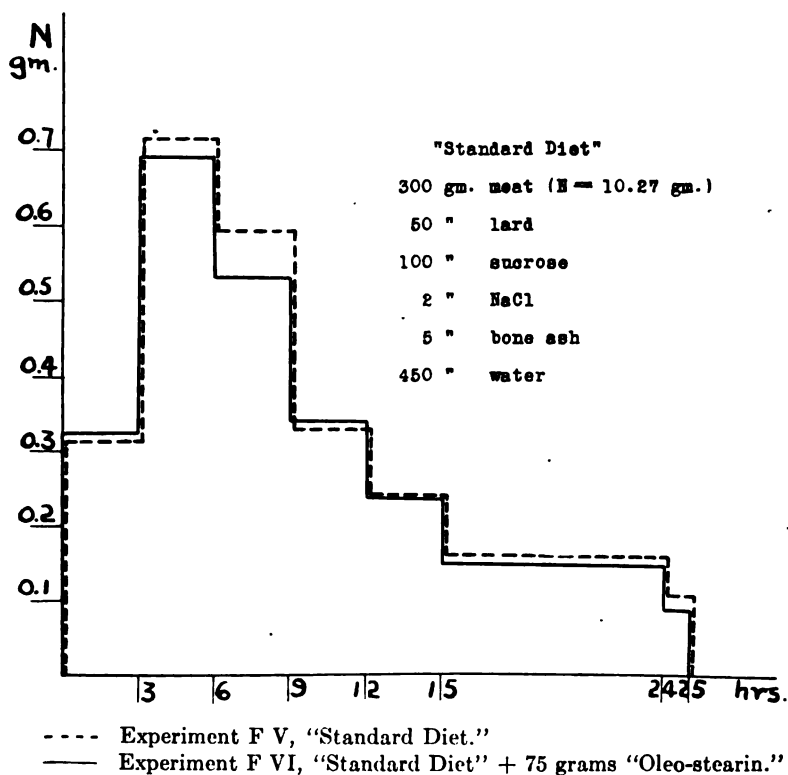
CURVE VII. To illustrate the effect on the rate of nitrogen elimination of substituting "Oleo-stearin" for the non-nitrogenous constituents of the "Standard Diet."



*"Oleo-stearin" (Addition) (Curve VIII).*

In the above experiments with fats the sugar was completely removed from the diet. When fat was *superimposed* on the "Standard Diet," the sucrose thus being retained, a nitrogen-output curve of the same character as that occurring after the ingestion of the "Standard Diet" followed.

CURVE VIII. To illustrate the effect of an *addition* of "*Oleo-stearin*" to the "Standard Diet" on the rate of nitrogen elimination.

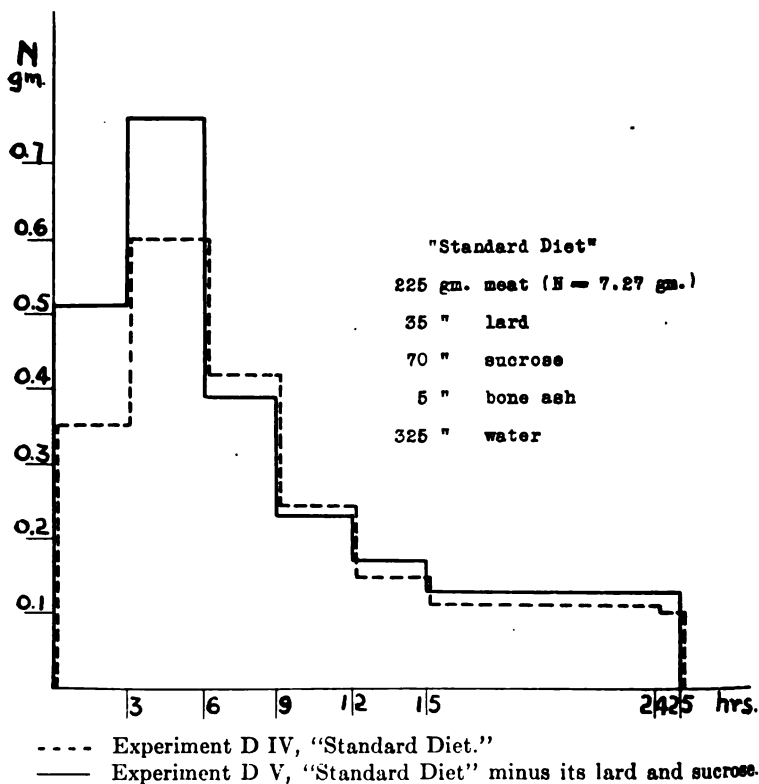


## EXPERIMENT WITHOUT FAT OR CARBOHYDRATE IN THE DIET.

(Curve IX.)

In the discussion which follows reference will be made to an experiment where meat alone was given, the "Standard Diet" minus its content of sucrose and lard being fed. In the first two periods the nitrogen output under these conditions was larger than when the "Standard Diet" was fed intact; in the later periods, practically identical with the "standard."

CURVE IX. Comparison of the rate of nitrogen elimination after the ingestion of a mixed diet with that when meat alone is fed.



## DISCUSSION.

The substitution of the different fats for the non-nitrogenous constituents of the "Standard Diet" did not yield concordant results. With cotton-seed oil the nitrogen-output curve was practically identical with the "standard;" with lard and "Oleo-stearin," higher in the first periods and afterwards the same as when the "Standard Diet" was fed. In these substitution experiments with fats two important changes in the diet were made: (a) a larger amount of fat was given; (b) carbohydrate was removed. One or both of these two changes must be responsible for the results obtained. The experimental data show that the typical "standard" curve was obtained when a large quantity of "Oleo-stearin" was *superimposed* on the "Standard Diet." Therefore, the larger amount of fat was not the important factor in causing the initial rise of the nitrogen-output curve above the normal when the non-nitrogenous constituents of the diet were replaced by "Oleo-stearin" (and *a priori* by lard). Furthermore, the nitrogen-output curve following the ingestion of the "Standard Diet" minus its content of sucrose and lard shows a close similarity to those obtained when lard and "Oleo-stearin," respectively, replaced the non-nitrogenous constituents of the "Standard Diet." In both types of experiment the sucrose of the "Standard Diet" has been removed. From the results obtained with carbohydrates<sup>8</sup> it is evident that sucrose in the diet exerts a retarding influence on nitrogen excretion. It is not surprising, then, that with removal of the sucrose there was an increased output of nitrogen in the early periods. Obviously lard and "Oleo-stearin" *per se* have no influence on the rate of elimination of nitrogen.

With cotton-seed oil there was a slight preliminary delay in the nitrogen excretion. In the light of the foregoing discussion the effect of this fat must have been much more pronounced than is evident from the data. With the removal of the sucrose of the "Standard Diet" there would be a tendency for a more rapid elimination of nitrogen in the early periods. The fact that there is, on the contrary, a slower rate can only be explained by assuming that the cotton-seed oil has caused a marked retardation of the nitrogen excretion. This is made the more evident by contrasting

<sup>8</sup> See the first part of this paper.

the nitrogen-output curve obtained when cotton-seed oil was present in the diet with that when meat alone was fed (cf. Curves V and IX).

The results with cotton-seed oil are in accord with those of earlier investigators who studied the influence of fat on the nitrogen-output curve. Panum (1874) and Feder (1881) found that lard caused a delay in the nitrogen excretion. Pari (1908) and Levene and Kober (1909) confirmed these findings; and Vogt (1906) likewise reported that fat (no mention of its character is made) caused a flattening of the nitrogen-output curve. All of these investigators used dogs as subjects of experimentation.<sup>9</sup> The discrepancy between the results of the experiments with lard here reported and those of former workers awaits an explanation.

As causes of the retardation of the rate of nitrogen elimination on the part of cotton-seed oil there are several possibilities, viz: a delay in gastric discharge, a subnormal rate of absorption, altered metabolic conditions. The reports of Cannon<sup>10</sup> that fat causes a retardation of gastric discharge, and of Vogt (1906) and Boettcher and Vogt (1909) that fat in the diet leads to a delayed absorption, make it quite probable that a sub-normal rate of alimentation is the prime cause of the results with cotton-seed oil; for no evidence has been found that altered metabolic conditions play a part in this connection.

The lack of concordance in the results obtained with the different fats may be explained by the fact that cotton-seed oil becomes more thoroughly incorporated in the diet and thus has a greater effect on the processes of alimentation, and hence on the nitrogen-output curve, than do the solid fats. An example of how varied the behavior of fats of different textures in the diet may be is afforded by experiments of Tangl and Erdélyi,<sup>11</sup> showing that the rate of discharge of fat from the stomach is dependent to a great extent upon its melting point and viscosity.

<sup>9</sup> Although the results have no bearing on the experiments here reported, it should be noted that Wolf (1912) studied the effect on the nitrogen-output curve of feeding fat to a fasting man.

<sup>10</sup> Cannon: *Amer. Journ. of Physiol.*, xii, p. 387, 1904.

<sup>11</sup> Tangl and Erdélyi: *Biochem. Zeitschr.*, xxxiv, p. 94, 1911.

## SUMMARY OF RESULTS WITH FATS.

The effect on the nitrogen-output curve of replacing the non-nitrogenous constituents of a mixed diet by fat varied with the character of the fat as follows: (a) with the fluid cotton-seed oil there was a slower rate of nitrogen elimination; (b) with lard and "Oleo-stearin" the nitrogen excretion in the early periods following the meal was above the normal. The apparent action of these latter fats was shown to be in reality the result of removing the sucrose from the diet.

The action of cotton-seed oil *per se* was to cause a marked delay in the rate of elimination of nitrogen. Neither lard nor "Oleo-stearin" by themselves had any effect on the nitrogen-output curve.

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## THE RATE OF ELIMINATION OF NITROGEN AS INFLUENCED BY DIET FACTORS.

### III. THE INFLUENCE OF THE CHARACTER OF THE INGESTED PROTEIN.

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(Received for publication, August 4, 1913.)

The influence which the character of the protein intake has on the rate of nitrogen elimination may be considered to better advantage now that the effect of various other diet factors has been determined.<sup>1</sup> The fact that there has been considerable discussion in recent years as to whether or not all proteins are catabolized with equal rapidity gives stimulus to further research on this subject.

In comparison with previous experiments the "Standard Diet" used in the present study contained a small amount of water;<sup>2</sup> and the relative amounts of fat and carbohydrate were changed.<sup>3</sup> With this low water intake two of the animals invariably gave an atypical "standard" curve of nitrogen output, the maximum excretion not occurring until the third three-hour period instead of in the second.<sup>4</sup> The fact that there was a delay in nitrogen excretion when these animals were given the modified "Standard

<sup>1</sup> Cf. Mendel and Lewis: this *Journal*, xvi, pp. 19 and 37, 1913.

<sup>2</sup> The proteins were all in the form of dry powders and, if an amount of water as large as had been given were now used, it was thought that there would be difficulty in getting the animal to eat the entire ration.

<sup>3</sup> Inasmuch as carbohydrates have been shown to exert a retarding influence on the rate of nitrogen elimination (cf. Mendel and Lewis: this *Journal*, xvi, p. 37, 1913), it seemed advisable to reduce the amount of carbohydrate in the diet. Except for the changes in the "Standard Diet" we employed the methods of our first paper (this *Journal*, xvi, p. 19, 1913).

<sup>4</sup> Cf. Mendel and Lewis: this *Journal*, xvi, p. 23, 1913.

Diet" could not be explained by the change in the relative amounts of fat and sugar in this diet.<sup>5</sup> The only other explanation, then, was that the variation was due to the decrease in the water intake. This possibility was suggested by the comparatively low volume of urine per kilo of body weight of these atypical animals. In order to determine whether this hypothesis was correct, an experiment was conducted on one of these animals in which the water content of the diet was made proportional to that of meat.<sup>6</sup> This time the typical "standard" curve with the maximum output of nitrogen in the second three-hour period was obtained. Thus it is conclusive that too great a diminution in the intake of water will cause a marked slowing of the rate of elimination of nitrogen in the urine. In the experiments to be reported the results are in all cases compared with those obtained on the same animal after the ingestion of this modified diet, a fact which the reader should bear in mind.

#### PRELIMINARY EXPERIMENTS.

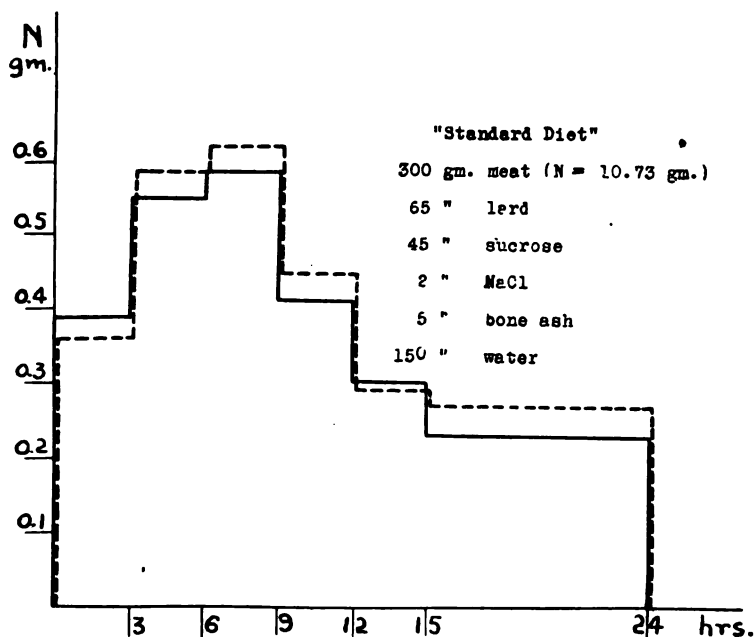
##### *Dried meat (Curve I).*

In the present study the rate of elimination of nitrogen was determined after the ingestion of several different proteins, the majority of which were in the form of dry powders. The difference in texture between such dry preparations and moist meat suggested itself as a possible cause for a variation in the nitrogen-output curve and so demanded a preliminary investigation. For this purpose a day's portion of meat was dried at about 55°C., then finely ground in a coffee mill, and fed. The drying of meat had practically no effect on the rate of nitrogen elimination.

<sup>5</sup> In a previous paper (this *Journal*, xvi, p. 37, 1913) the authors have shown that carbohydrate in the diet causes a delay in nitrogen excretion. The reduction of the amount of carbohydrate in this case would tend to have an opposite effect.

<sup>6</sup> The water addition in our original "Standard Diet" was calculated on this basis (cf. this *Journal*, xvi, p. 19, 1913).

CURVE I. To illustrate the absence of effect on the rate of elimination of nitrogen of previously *drying* the meat of the "Standard Diet."



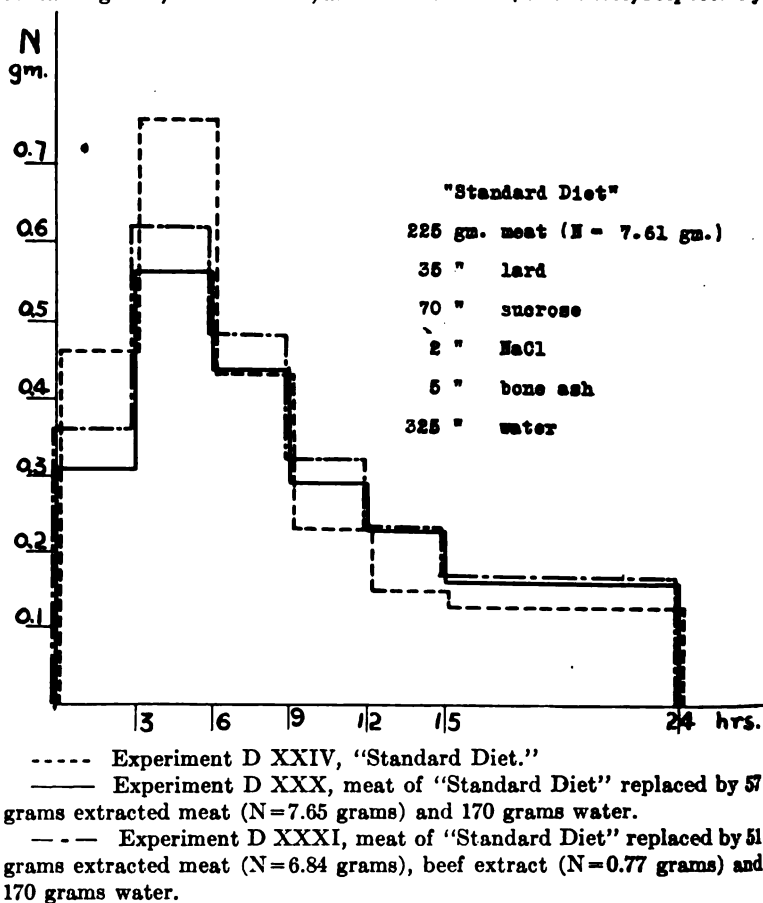
----- Experiment G XVIII, "Standard Diet."  
 ——— Experiment G XX, "Standard Diet," except that meat had been previously dried (dry weight = 75 grams) and finely ground. Extra water = 225 cc.

#### *Extracted meat*<sup>7</sup> (Curve II).

Inasmuch as a notable proportion of the nitrogen of meat is non-protein in character, it seemed desirable to study meat devoid of extractives and in such a form as to be directly comparable with the extractive-free isolated proteins. The nitrogen-output curves of the latter are compared with that occurring after the ingestion of an extracted meat powder. The use of such an extractive-free meat is made the more necessary because of the recognized influence of extractives on secretory processes in the stomach, and because the nitrogen of extractives like creatine and the purines is in a different chemical structure from that

<sup>7</sup> A light brown, impalpable powder obtained from Armour and Company.

CURVE II. Comparison of the rates of nitrogen elimination on diets containing *meat*, *extracted meat*, and *extracted meat+extractives*, respectively.



of the familiar amino-acids. The nitrogen-output curve with the extracted meat was considerably more flattened than that after the ingestion of fresh meat—a fact which may be explained in part by the absence of extractives in the former product, the following experiments showing that extractive-nitrogen is very rapidly eliminated.

*Extracted meat + Extractives (Curve II).*

When extracted meat furnished 90 per cent, and Liebig's beef extract 10 per cent of the nitrogen of the diet, there was a larger nitrogen output in the first periods than after the ingestion of extracted meat alone, showing that extractive-nitrogen is eliminated with comparative rapidity.

*Meat + Urea (Curve III).*

When the nitrogen of the diet was furnished in equal portions by meat and urea, the nitrogen excretion in the first two periods was enormously larger than when meat alone was fed. It is evident that the urea-nitrogen is rapidly eliminated.<sup>8</sup>

In the first of these two experiments (Curve II) extractive-nitrogen was present in approximately the same proportion as it occurs in meat; the nitrogen-output curve, however, was considerably more flattened than when fresh meat was fed. It is quite apparent, therefore, that the absence of extractives can only account in part for the slower rate of nitrogen elimination when extracted meat replaces the meat of the "Standard Diet." The cause of this difference between the nitrogen-output curve of fresh meat and that of extracted meat may be that the latter product is richer in connective tissue and so less readily digestible. The finding of Mendel and Fine<sup>9</sup> that this same extracted meat was not as well utilized as fresh meat bears out such an assumption.

## PROTEIN MATERIALS EMPLOYED BESIDES MEAT.

In several cases the materials used in the present study were isolated proteins; in others, products relatively rich in protein. A description of each of the substances used follows.

Casein<sup>10</sup>—a purified preparation in the form of an impalpable powder containing 13.36 per cent nitrogen.

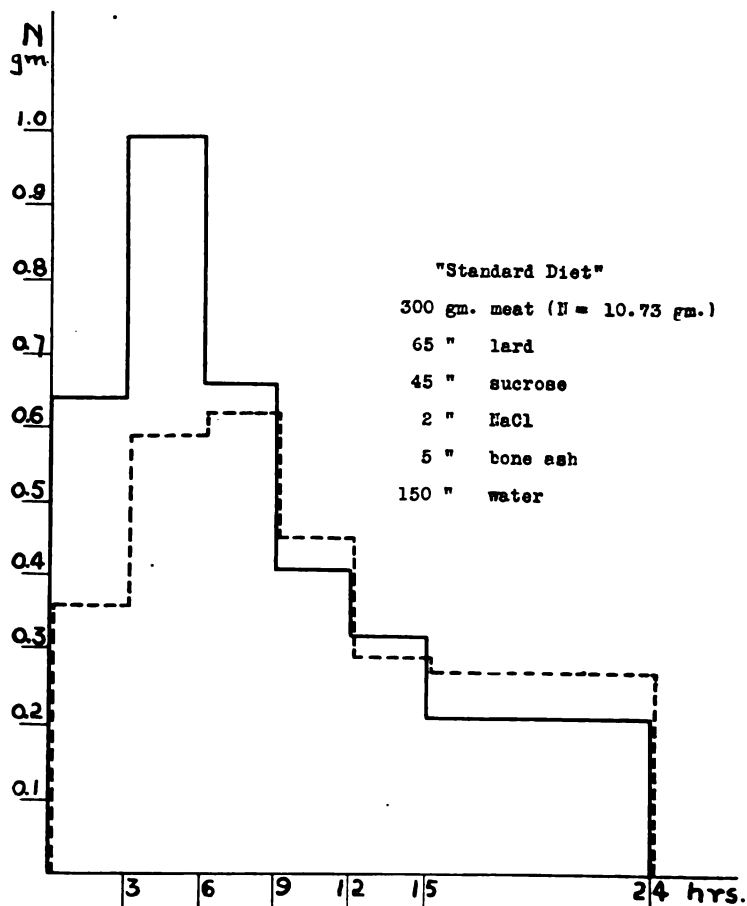
<sup>8</sup> Wolf (1912b) and Cathcart and Green (1913) have reported a rapid elimination of nitrogen after feeding urea to man.

<sup>9</sup> Mendel and Fine: this *Journal*, xi, p. 5, 1912.

<sup>10</sup> The casein and a portion of the edestin were contributed by Dr. T. B. Osborne, New Haven, Conn.

## Rate of Nitrogen Elimination

CURVE III. To illustrate the effect on the rate of elimination of nitrogen of replacing one-half of the meat of the "Standard Diet" by *urea*.



----- Experiment G XVIII "Standard Diet."

———— Experiment G XXII, one-half of meat of "Standard Diet" replaced by 11.5 grams of urea (N=5.37 grams) and 115 grams of water.

Ovovitellin<sup>11</sup>—a purified product; an impalpable powder containing 13.78 per cent nitrogen.

Edestin—a purified preparation. Two lots of this material were used, both of which were impalpable powders containing 16.23 per cent and 16.95 per cent of nitrogen, respectively.

"Glidine"<sup>12</sup>—a commercial preparation from wheat;<sup>13</sup> an impalpable powder giving no starch reaction and containing 14.8 per cent nitrogen.

Gelatin—a commercial preparation in the form of a fine powder containing 15.1 per cent nitrogen.

Soy Bean<sup>14</sup>—an impalpable powder containing 7.25 per cent nitrogen, thus being poor in protein as compared with the other dry materials used. Besides protein the soy bean contains a large amount of fat and considerable cane sugar.<sup>15</sup>

Liquid Egg-White—the whites of eggs thoroughly strained and mixed (nitrogen content=1.95 per cent).

Dried Egg-White—the whites of eggs dried at 50°C. and then ground to a fine powder in a mortar (nitrogen content=13.6 per cent).

Coagulated Egg-White—the whites of hard boiled eggs passed through a sieve (nitrogen content=1.92 per cent).

Ovalbumin<sup>16</sup>—a purified product in the form of an impalpable powder containing 15.4 per cent nitrogen.

Water was added to the powdered proteins (with the exception of gelatin, dried egg-white, and ovalbumin) the night previous to feeding, for the purpose of allowing ample time for "hydration" of the material. The following morning a thoroughly hydrated mush was always found.

<sup>11</sup> Prepared by Mr. R. L. Kahn in our laboratory.

<sup>12</sup> Obtained from Menley and James, New York City.

<sup>13</sup> This material, "according to Bergell, and Thiemar, is prepared from wheat flour by a process of washing and centrifuging."

<sup>14</sup> Mr. M. F. Deming of the Cereo Company, Tappan, New York, contributed this material.

<sup>15</sup> For a complete analysis of soy bean, see Ruhräh: *Journ. Amer. Med. Assn.*, liv, p. 1664, 1910; also quoted by Mendel and Fine: this *Journal*, x, p. 435, 1911.

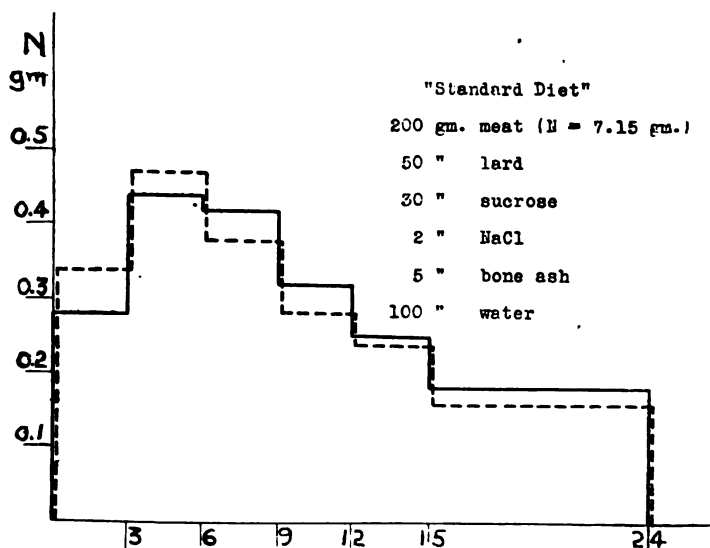
<sup>16</sup> This material was prepared in our laboratory by Dr. Martha Tracy.

## EXPERIMENTS WITH PROTEINS.

*Casein* (Curve IV).

When casein was substituted for the meat of the "Standard Diet," the nitrogen-output curve was practically the same as that with extracted meat.

CURVE IV. Comparison of the rates of nitrogen elimination on diets containing *extracted meat* and *casein*, respectively.



----- Experiment H VII, meat of "Standard Diet" replaced by 53 grams extracted meat (N=7.11 grams) and 150 grams water.

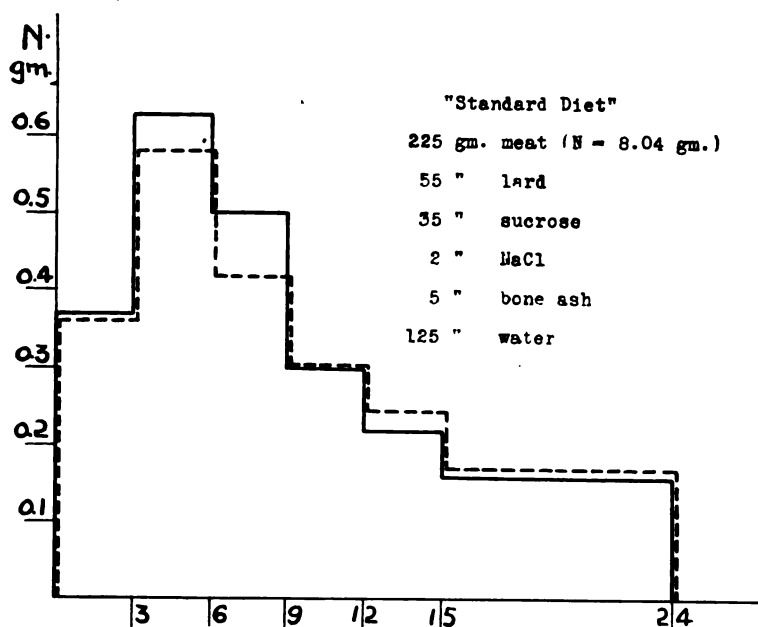
———— Experiment H VIII, meat of "Standard Diet" replaced by 54 grams casein (N=7.21 grams) and 150 grams water.



*Ovovitellin* (Curve V).

The rate of nitrogen elimination after the ingestion of ovovitellin was identical within the limits of experimental error with that when extracted meat was fed.

CURVE V. Comparison of the rates of nitrogen elimination on diets containing *extracted meat* and *ovovitellin*, respectively.



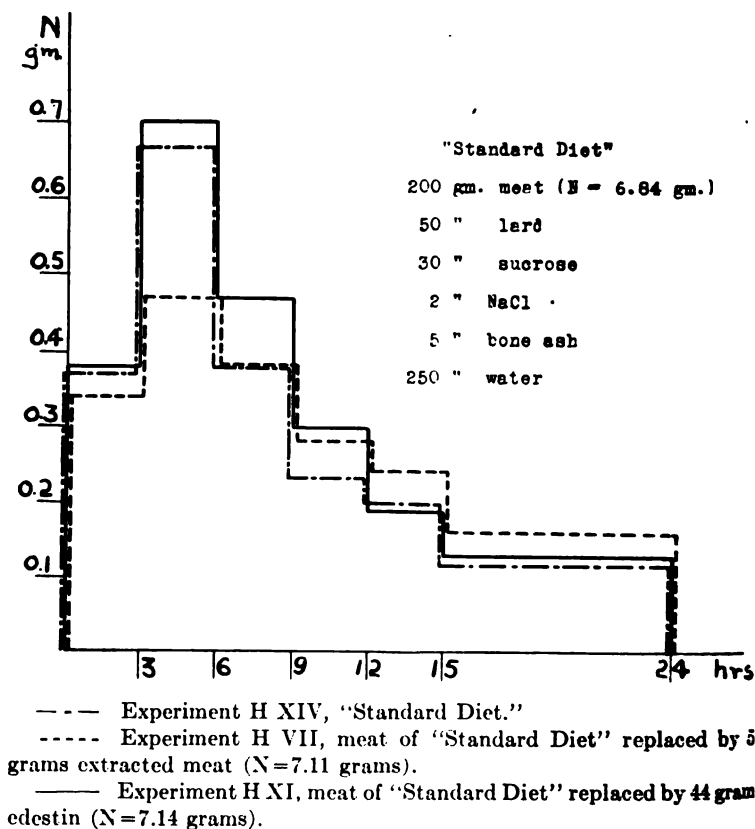
----- Experiment D XXXIII, meat of "Standard Diet" replaced by 60 grams extracted meat (N=8.05 grams) and 170 grams water.

———— Experiment D XXXVIII, meat of "Standard Diet" replaced by 58 grams ovovitellin (N=7.99 grams) and 170 grams water.

*Edestin* (Curve VI).

The nitrogen-output curve with edestin was not of such a flattened aspect as that with extracted meat. The nitrogen excretion in the earlier periods was larger; in the later periods, small than with extracted meat. The edestin curve, however, was very much the same as that with fresh meat.

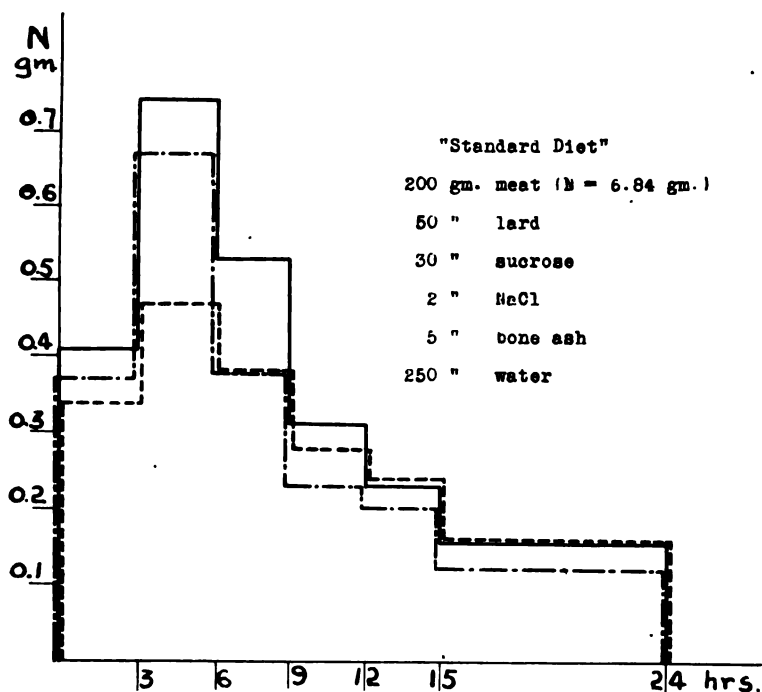
CURVE VI. Comparison of the rates of nitrogen elimination on diet containing meat, extracted meat, and edestin, respectively.



*"Glidine"* (Curve VII).

When "*Glidine*" constituted the protein intake, the nitrogen excretion was larger during the earlier periods of the day than with extracted meat. The character of the nitrogen-output curve was practically the same as that with fresh meat; the two curves ran parallel, that with "*Glidine*" being at a higher level.

CURVE VII. Comparison of the rates of nitrogen elimination on diets containing *meat*, *extracted meat*, and "*Glidine*," respectively.

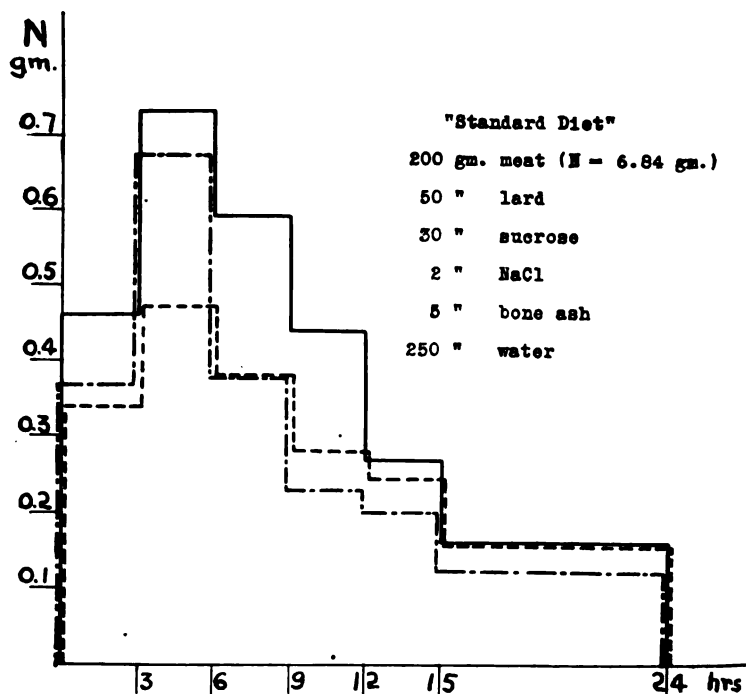


--- Experiment H XIV, "Standard Diet."  
 ---- Experiment H VII, meat of "Standard Diet" replaced by 53 grams extracted meat (N=7.11 grams).  
 — Experiment H IX, meat of "Standard Diet" replaced by 48 grams "*Glidine*" (N=7.10 grams).

*Gelatin (Curve VIII).*

Again with gelatin the nitrogen-output curve was not as flattened as that with extracted meat, but identical in character with the "standard" (fresh meat) curve. There was a negative balance with gelatin and the nitrogen excretion for all the period was higher than with meat.

CURVE VIII. Comparison of the rates of nitrogen elimination on diets containing *meat*, *extracted meat*, and *gelatin*, respectively.

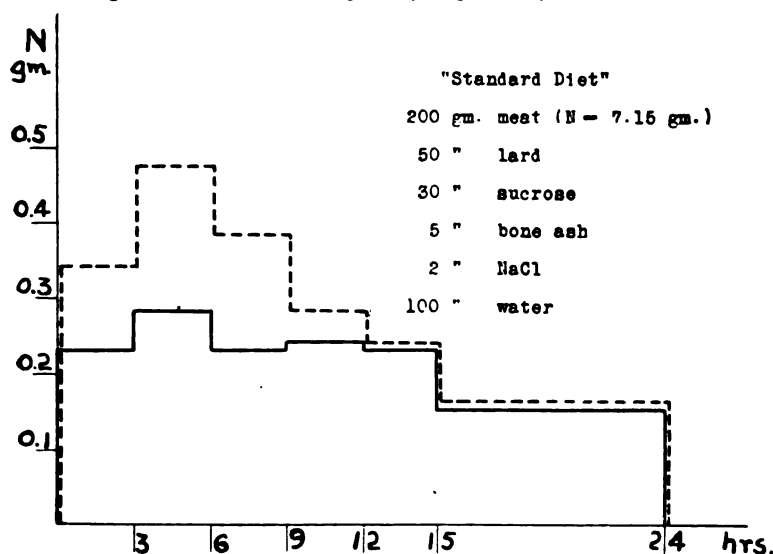


- Experiment H XIV, "Standard Diet."  
 ---- Experiment H VII, meat of "Standard Diet" replaced by 100 grams extracted meat (N=7.11 grams).  
 - · - Experiment H X, meat of "Standard Diet" replaced by 47 grams gelatin (N=7.08 grams).

*Soy bean (Curve IX).*

On account of the comparatively poor utilization of soy bean<sup>17</sup> its ingestion was followed by a smaller nitrogen output in all the periods of the day than when extracted meat was fed. Furthermore, this lower curve was not parallel to that with extracted meat; its character was quite different. In the earlier periods of the experiment here reported a smaller percentage of the 24-hour nitrogen output was excreted than when meat was fed; in the later periods, a larger percentage. In a second experiment the maximum nitrogen excretion did not occur until the third three-hour period, instead of the second. In both cases, then, there was a delay in the elimination of nitrogen independent of the poorer utilization of the soy bean.

CURVE IX. Comparison of the rates of nitrogen elimination on diets containing *extracted meat* and *soy bean*, respectively.



----- Experiment H VII, meat of "Standard Diet" replaced by 53 grams extracted meat (N=7.11 grams) and 150 grams water.

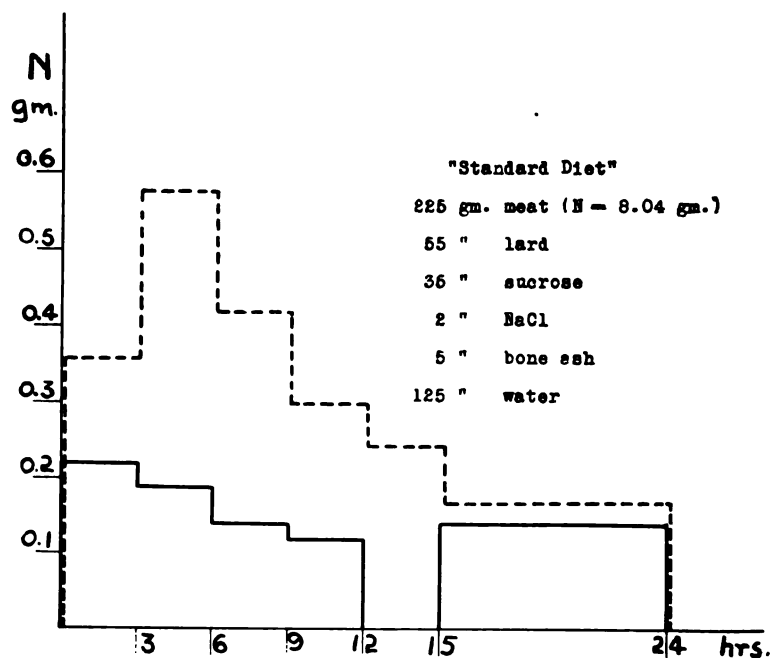
———— Experiment H XII, meat of "Standard Diet" replaced by 99 grams soy bean (N=7.18 grams) and 150 grams water.

<sup>17</sup> Mendel and Fine (this *Journal*, x, p. 345, 1911) found soy bean nitrogen to be poorly utilized.

*Uncoagulated egg-white (Curve X).*

On the days when native egg-white was fed the nitrogen output was only about half as large as the intake. It is probable, as the discussion to follow will show, that this material was poorly utilized. The character of the nitrogen-output curve with uncoagulated egg-white was somewhat different from that with extracted meat, the maximum excretion occurring during an earlier period—in two experiments during the first three-hour period,

CURVE X. Comparison of the rates of nitrogen elimination on diets containing *extracted meat* and *liquid egg-white*, respectively.



----- Experiment D XXXIII, meat of "Standard Diet" replaced by 60 grams extracted meat (N=8.05 grams) and 170 grams water.

———— Experiment D XXXII, meat and water of "Standard Diet" replaced by 412 cc. liquid egg-white (N=8.03 grams).<sup>18</sup>

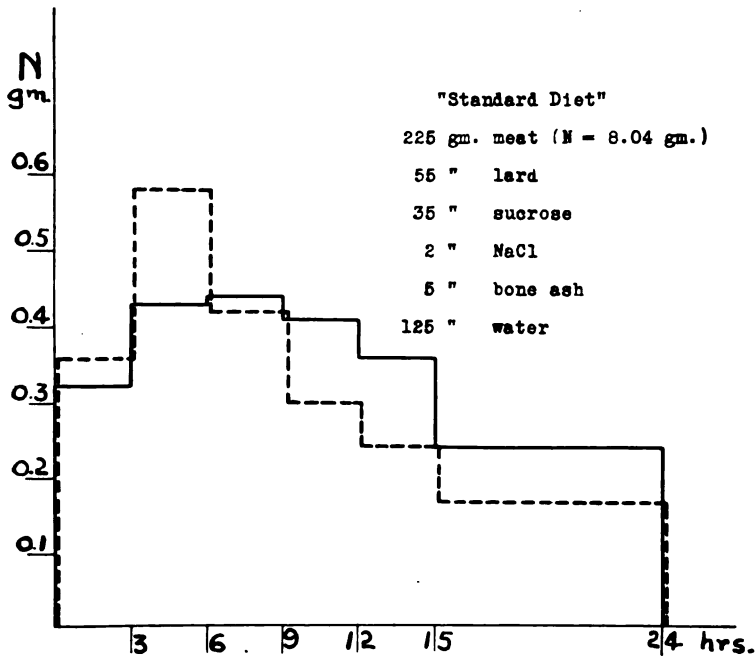
<sup>18</sup> Diarrhea during 5th three-hour period; urine contaminated. The ingestion of dried native egg-white was also followed by diarrhea.

instead of during the second; in a third experiment during the second period, instead of during the third. The results with liquid egg-white and dried egg-white, respectively, were concordant.

*Coagulated egg-white (Curve XI).*

Coagulated egg-white was evidently well utilized; but the nitrogen-output curve after its ingestion was more flattened than that when extracted meat was fed. In other words there was a comparative delay in the elimination of nitrogen when coagulated egg-white constituted the protein intake.

CURVE XI. Comparison of the rates of nitrogen elimination on diets containing *extracted meat* and *coagulated egg-white*, respectively.



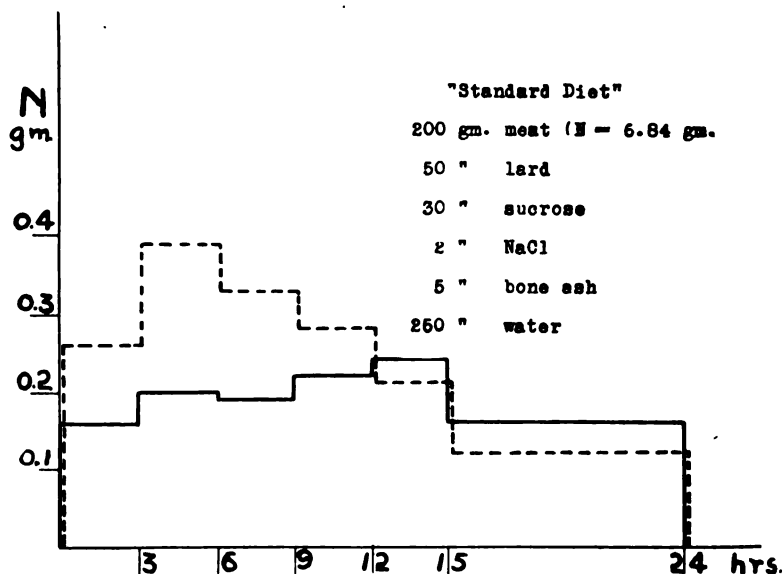
----- Experiment D XXXIII, meat of "Standard Diet" replaced by 60 grams extracted meat (N=8.05 grams) and 170 grams water.

———— Experiment D XXXVII, meat of "Standard Diet" replaced by 419 grams coagulated egg-white (N=8.04 grams).

*Ovalbumin*<sup>19</sup> (Curve XII).

When ovalbumin was fed the urinary nitrogen-output was much smaller than the intake, the marked diarrhea about nine hours after the meal suggesting a poor utilization of the ovalbumin as the cause of the smaller nitrogen excretion. Furthermore, the rate of nitrogen elimination with this material was quite different from that with extracted meat. There was a rise to a maximum in the second three-hour period, then a slight fall during the third three hours, followed by a second rise to a maximum in the fifth

CURVE XII. Comparison of the rates of nitrogen elimination on diets containing *extracted meat* and *ovalbumin*, respectively.



<sup>19</sup> We are greatly indebted to Mr. R. L. Kahn for performing this and several other experiments of the present series.

<sup>20</sup> Voluminous diarrhea during third three-hour period; animal ate some feces.



period. The fact that the animal ate a portion of the diarrheal feces at the beginning of the third period may account in part for the second rise.

#### DISCUSSION.

The nitrogen-output curves following the ingestion of unaltered meat and extracted meat powder, respectively, were quite different. The slower rate of elimination of nitrogen when extracted meat was fed cannot be explained by the dry condition of this material; for the nitrogen-output curve was unchanged by a previous drying of the meat of the "Standard Diet." The absence of extractives in the extracted meat can only account in part for the delayed nitrogen excretion. This product presumably contains proportionately more connective tissue than the fresh meat used and thus is digested more slowly. The nitrogen-output curves following the ingestion of casein and ovovitellin are practically identical with that of extracted meat; the character of the curves with edestin, "Glidine," and gelatin is the same as that with fresh meat.

Soy bean, egg-white, and isolated ovalbumin gave nitrogen-output curves radically different from those of either of the meat products studied. The comparative delay in nitrogen elimination independent of the poor utilization of soy bean may be explained in part by a greater difficulty of digestion of this product, and in part by the presence of sucrose in soy bean, it having been shown in a previous paper<sup>21</sup> that the presence of carbohydrate in the diet delays nitrogen excretion. The comparatively small excretion of nitrogen after the ingestion of native egg-white and ovalbumin is caused in all probability by a poor utilization of these materials, the early diarrhea following their ingestion making such an explanation quite likely. That uncoagulated ovalbumin is poorly utilized was reported by Falta (1906), who found that the coefficient of utilization of this material in man was only about 70 per cent. Wolf (1912b) fed a large quantity of native egg-white to man and reported that only about half was utilized. When liquid egg-white<sup>22</sup> was fed it is probable that very little gastric proteolysis occurred;

<sup>21</sup> Mendel and Lewis: this *Journal*, xvi, p. 37, 1913.

<sup>22</sup> The dried egg-white mixed with water would be essentially the same as the natural product.

for Cannon,<sup>21</sup> and London and Sulima,<sup>22</sup> working with cats and dogs, respectively, have reported that this material begins to leave the stomach almost immediately after ingestion. The early discharge of the stomach, the comparatively early emptying of the bowel, and the unfavorable character of liquid egg-white for the action of digestive enzymes, together with a possible resistance of native protein to digestion, may all contribute to a poor utilization of this material. Coagulated egg-white was well utilized; but following its ingestion there was a comparative delay in nitrogen excretion. The slower rate of elimination of nitrogen with this source of protein cannot be accounted for by a delay of gastric discharge; for Cannon, and London and Sulima have demonstrated that egg-white coagulated by heat leaves the stomach more rapidly than most proteins. It is probable that the delayed excretion of nitrogen may be caused in part, at least, by a comparative difficulty of digestion of coagulated egg-white on account of the compact and impermeable character of the fine particles of coagulum.

A few reports in the literature are in harmony with this view that such changes as do occur in the rate of elimination of nitrogen after the ingestion of different protein materials may be explained by variations in alimentary, rather than metabolic processes. Van Slyke and White (1911), using the method of the present work, demonstrated that different nitrogen-output curves were obtained after feeding various boiled fish meats to a dog; and attributed this result to a variation in the rate of digestion. The validity of such an explanation is made very clear by a comparison of the results obtained by these authors with fresh and salt cod, there being as much difference in the nitrogen-output curves of the two preparations of this one fish as in those of any of the different fish. Vogt (1906) investigated the effect on the rate of elimination of nitrogen of superimposing various proteins in considerable quantities on a standard diet, finding that both coagulated and uncoagulated ovalbumin caused a delay in nitrogen excretion whereas edestin and a casein preparation (Nutrose) gave a nitrogen-output curve of approximately the same character

<sup>21</sup> Cannon: *Amer. Journ. of Physiol.*, xii, p. 387, 1904.

<sup>22</sup> London and Sulima: *Zeitschr. f. physiol. Chem.*, xlii, p. 232, 1905.

as meat. This author believed that the delay with ovalbumin was caused by a comparatively slow rate of digestion of this material. Loeb (1911) studied the rate of elimination of nitrogen after replacing about one-half of the meat of a standard diet by another form of protein; and demonstrated that there was only a very slight change when meat and casein, respectively, were fed, although considerable difference existed between the curves of these proteins on the one hand and those of their hydrolyzed products on the other. In experiments where the urine was collected only for twelve-hour periods Falta, Grote, and Staehelin (1907) found approximately the same rate of nitrogen excretion with casein as with meat. All of these investigators worked on dogs. Wolf (1912a, 1912b) added various proteins and non-proteins to a "standard" diet in man and collected the urine in hourly periods, studying among other things the rate of elimination of nitrogen. He found little difference in the nitrogen-output curves following the ingestion of gelatin and plasmon, respectively. With veal, however, there was a somewhat slower rate of nitrogen output. Native egg-white and coagulated egg-white gave results quite similar to those reported in the present paper. Wolf (1912c) also studied the rate of nitrogen elimination in dogs after feeding cooked and raw meat, respectively, obtaining practically identical results in the two cases.

In considering the significance of the results of the present study attention should be given to Falta's conclusions from his work on the rate of metabolism of proteins. The method of investigation employed by this author was to determine the average daily nitrogen-output of a dog in nitrogen equilibrium for a period of several days, then to superimpose the protein to be studied on the "standard" diet, and to ascertain how long a time was required for a reappearance of an excess of nitrogen in the urine equivalent to the nitrogen of the superimposed material. Falta (1904 and 1906) studied different proteins on man and found that with most of these more than half of the excess nitrogen reappeared on the first day, about three days being required for the entire amount to show up. With casein, for example, about two-thirds of the excess nitrogen reappeared during the first day, and most of the remainder on the second day. A few exceptions occurred, however,

the most striking being with ovalbumin and ovovitellin.<sup>23</sup> In these cases only about 27 per cent of the excess nitrogen appeared the first day; and five days were required for all to reappear, although all but a very small amount had come out in three days. When coagulated ovalbumin was the added protein no longer time was required for the reappearance of the excess nitrogen than was the case with casein. With dogs the results with ovalbumin and casein were the same as for casein with man. These observations on man were confirmed by Hämäläinen and Helme (1907), who demonstrated that a longer time was required for the reappearance of the excess nitrogen with egg-white than with a casein preparation (Proton) or roast veal. Cathcart and Green (1913), employing the Falta method of superimposition on man, reported that with egg-white, both coagulated and uncoagulated, only a small part of the extra nitrogen appeared in the urine even after several days. There was little difference in the rate of elimination of the extra nitrogen after adding veal and gelatin, respectively, to the diet, greater differences being obtained with the same sample of gelatin in two experiments where the basal rations varied considerably. Vogt (1906) used Falta's method of study on dogs and found that a longer time was required for the reappearance of the excess nitrogen when egg-white, both uncoagulated and coagulated, was superimposed on the standard diet than when edestin or a casein preparation, Nutrose, was added. All of these communications are in harmony with that of Graffenberger (1891), who showed by a somewhat different method that when gelatin or fibrin was superimposed on a standard diet the excess nitrogen reappeared more rapidly than when peptone constituted the increased nitrogen intake.

From the results of his experiments Falta (1906) has concluded that the longer time required for the reappearance of the excess nitrogen after superimposing ovalbumin on the standard diet was the result of the absorption of comparatively large cleavage products of this protein, a longer time being required for the catabolism of these higher protein residues. Hämäläinen and Helme (1907) held a similar view; and Levene (1909a, 1909b, 1909c, 1910) and his co-workers from a series of studies of an entirely different

<sup>23</sup> Only one experiment with ovovitellin is reported and the author says that no definite conclusion should be drawn from a single experiment.

nature likewise came to the conclusion that the higher protein cleavage products are catabolized more slowly than the simple amino-acids. Vogt (1906) was not inclined to favor such an explanation, maintaining that a slower rate of digestion and absorption might account in part for the results obtained by him with egg-white, and that certain unknown factors of intermediary metabolism might play a part.

Although Falta's experiments are not directly comparable with those of the present study, yet if one recalls how readily texture of the diet influences the rate of digestion and absorption independently of the character of the protein, it seems quite likely that Falta's results were caused in part by a difference in the rate of digestion of the various materials studied. Let us consider what would be the effect of a markedly delayed digestion and absorption in studies of the type that Falta made. In the experiments of this author on man the superimposed protein as well as the standard diet was fed in four portions distributed over the day. Under such conditions it is quite likely that absorption of the digestion products of a difficultly digestible protein would not be complete until after the beginning of the second day. It is not surprising, then, that the excess nitrogen eliminated on the day when the protein was added to the diet should be smaller than when the superimposed protein was readily digestible; nor that it should be greater on the following day, the amount of nitrogen absorbed on this second day being greater than that usual on a normal day. The fact that, when Falta fed a single meal to dogs at the beginning of the day, he obtained a result with ovalbumin similar to that with casein makes such an explanation more probable; for in this case digestion and absorption would certainly be complete during the first day.

#### SUMMARY OF RESULTS WITH PROTEINS.

The nitrogen-output curves following the ingestion of meat and extracted meat, respectively, differ considerably, that with the latter product being more flattened. This slower rate of elimination of nitrogen cannot be explained by the dried condition of the extracted meat; and only in part by the absence of extractives in this material. It is suggested that the extracted meat may

have contained proportionately more connective tissue than the fresh meat used and thus have been less readily digestible.

The nitrogen-output curves following the ingestion of most of the proteins studied—casein, ovovitellin, edestin, "Glidine," gelatin—differ in character to no greater extent than those obtained by feeding the two meat products employed. With egg-white, ovalbumin, and soy bean, however, curves of a character radically different from that of either of the meats were obtained. These results may be explained to a great extent by a difference in the rate and completeness of digestion and absorption of these materials; while the sucrose in the soy bean will also account in part for the delay in nitrogen elimination with this product. When these alimentary differences are duly taken into account, the conclusion seems justified that proteins do not differ materially in their rate of metabolism.

Falta's conclusions from his work on the rate of protein metabolism and the similar conclusions of Hämäläinen and Helme, and of Levene and his co-workers were discussed. From the results of the present study it seems quite probable that the findings of these authors may be explained by other factors than an assumed difference in the rate of metabolism of proteins caused by an absorption of larger or smaller cleavage products.

The results of the experiments reported in the papers of this series show that apart from the character of the protein ingested a large number of diet factors—the water intake, the presence and nature of indigestible materials in the diet, the amount and character of the carbohydrate fed, and to some extent the presence of fat in the diet—play a rôle in modifying the rate of elimination of nitrogen after a meal containing protein. With most of the proteins studied the nitrogen-output curves differed to only a slight extent from one another; and in no case did the nature of the protein have a greater effect on the rate of nitrogen elimination than some of the non-protein diet factors mentioned above.

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**THE CARBON DIOXIDE AND OXYGEN CONTENT OF THE  
BLOOD AFTER CLAMPING THE ABDOMINAL  
AORTA AND INFERIOR VENA CAVA  
BELOW THE DIAPHRAGM.**

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(Received for publication, August 22, 1913.)

In search of experimental support for the over-production theory of diabetes mellitus Porges<sup>1</sup> and Porges and Salomon<sup>2</sup> have found that ligation of the abdominal aorta and inferior vena cava just below the diaphragm, both in normal rabbits and in depancreatized dogs, causes a rise in the external<sup>3</sup> respiratory quotient. They interpret this result as proof (1) that the organism is dependent upon the liver for its power to oxidize protein and fat, carbohydrate only or carbohydrate chiefly being oxidized when the liver is excluded; and (2) that the depancreatized animal retains its power to oxidize sugar.

A rise in respiratory quotient after this radical interference with the circulation has been confirmed by Rolly,<sup>4</sup> who, however, finds the rise not at all constant and gives an altogether different explanation. Verzář<sup>5</sup> likewise witnessed a sudden change in the R. Q., but a change in the same direction, when the liver was partially excluded by anastomosis of the portal vein with the lower

<sup>1</sup> Porges: *Biochem. Zeitschr.*, xxvii, p. 131, 1910.

<sup>2</sup> Porges and Salomon: *Ibid*, p. 143.

<sup>3</sup> The term *external* R.Q. is used here in order to emphasize the fact that the exchange of gases between blood and outside air does not under all circumstances take place at the same rate as the exchange between blood and tissue. The volumetric relations between the CO<sub>2</sub> gain of venous blood and O<sub>2</sub> loss from arterial may be called the *internal* R.Q.

<sup>4</sup> Rolly: *Deutsch. Arch. f. klin. Med.*, cv, p. 494, 1912; *Munch. med. Wochenschr.*, 1912, Nos. 22 and 23.

<sup>5</sup> Verzář: *Biochem. Zeitschr.*, xxxiv, p. 52, 1912.

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part of the inferior vena cava (Queirolo operation). Fischler and Grafe<sup>6</sup> ligated the hepatic artery of dogs which, some weeks before, had been successfully operated for the Eck fistula, and found in two out of six cases a distinct rise in the R. Q. Böhm<sup>7</sup> reports but a very slight rise "after exclusion of the abdominal organs even in depancreatized dogs."

In all these experiments showing a higher R. Q.<sup>8</sup> there is, as would be expected, a reduction in the total respiratory exchange, depending in amount upon the kind and amount of tissue excluded from the circulation. The reduction in the absorption of oxygen is greater than that for the elimination of carbon dioxide. Hence the higher R. Q. In other words, after the crucial operation there is, relatively, a greater output of CO<sub>2</sub>.

There are the following possible ways of explaining this result: (1) A greater production of CO<sub>2</sub> with no change in the rate of elimination. If carbohydrate or carbohydrate-like bodies should be oxidized instead of protein or fat, more CO<sub>2</sub> would be produced. This is the explanation adopted by the von Noorden school. (2) Greater elimination of CO<sub>2</sub> from the blood with no essential change in the rate of production. The influences which may be conceived of as driving out more CO<sub>2</sub> may be (a) chemical or (b) mechanical. If more acids were produced, or if acids produced as usual were not neutralized, after exclusion of the liver, more CO<sub>2</sub> would be liberated from its combination in the tissues and the blood, and would escape. This is the explanation adopted by Rolly, and approved of by Fischler and Grafe. Rolly has actually found in the serum of his operated animals a lower degree of alkalescence than in that of normal animals and Porges in a recent paper<sup>9</sup> has himself shown that acidification of the blood by intravenous infusion of sodium dihydrogen phosphate will raise the respiratory quotient, though not so much as occurred in his earlier experiments.

The mechanical factors have not been sufficiently emphasized.

<sup>6</sup> Fischler and Grafe: *Deutsch. Arch. f. klin. Med.*, cviii, p. 516, 1912.

<sup>7</sup> Böhm: *Zentralbl. f. Physiol.*, xxvii, p. 120, 1913.

<sup>8</sup> Except one animal which had convulsions in Fischler and Grafe's series. Böhm's complete paper is not accessible and it is possible that his series may contain other exceptions.

<sup>9</sup> Porges: *Biochem. Zeitschr.*, xlvi, p. 1, 1912.

Porges, in his original article assumes that any change due to over-ventilation which might result would be equalized in fifteen minutes. Presumably he means by over-ventilation only exaggerated breathing for he cites in support of his view, the work of Bornstein and Gartzten<sup>10</sup> on the effects of over-ventilation by voluntary effort in human subjects, showing that *after fifty minutes no more CO<sub>2</sub> can be pumped out in this manner!* He also cites one of his own experiments in which the R. Q. in the second period was slightly higher than in the first period after ligation of the vessels. According to Porges' view, the quotient in the second period should be smaller if any factor of over-ventilation were operative.

Neither of these citations offers any convincing evidence which would exclude the mechanical factors; for in the experiments of Bornstein and Gartzten the circulation was in no way disturbed, while the forced breathing was maximal, and Porges' own experiment proves only that, whatever was the controlling cause of the higher quotient after ligation of the vessels, the conditions were the same in the second period as in the first. Moreover it should be borne in mind that over-ventilation may mean something more than exaggerated breathing: there may be over-aëration due solely to a disturbance to the pulmonary circulation.

Fischler and Grafe appreciated the possible effect of the disturbance to the circulation resulting from the Porges procedure, saying, "One does not know to what extent the results may be due to the direct consequences of this alteration."<sup>11</sup>

In the writers' opinion the work of Fischler and Grafe is sufficient refutation of the position taken by the von Noorden school as to the rôle of the liver in the metabolism of the food-stuffs. Excluding the liver by ligation of the hepatic artery after Eck fistula *did not cause a permanent rise in the respiratory quotient* in dogs which survived from six to twenty hours. On the other hand, there is no doubt, in certain cases at least, about the rise of quotient after ligation of the abdominal aorta and inferior vena cava just below the diaphragm. It remains to give a satisfactory explanation of this phenomenon.

It seems almost incredible that the purely mechanical effects of so radical a procedure as one which excludes at a stroke fully

<sup>10</sup> Bornstein and Gartzten: *Pflüger's Archiv*, cix, p. 628, 1905.

<sup>11</sup> Fischler and Grafe: *loc. cit.*, p. 519.

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one-half of the blood and considerably more than one-half of the animal's weight from the circulation should not have been more seriously considered. What effect would it have on the heart rate, on the blood pressure, on the rate of blood flow through the lungs? Neither Porges nor Porges and Salomon gives any data as to the pulse, blood pressure, rate of respiration or volume of respiration, to say nothing of the gaseous content of the blood before and after ligation of the vessels, and yet the results are presented as proof that, by turning a valve, so to speak, the metabolic processes are suddenly changed so that one fuel and one only can now supply the body's energies!

The first and most obvious control which one would think of in connection with so radical a change in the R. Q. would be the gaseous content of the blood. There are numerous experiments in the work of Zuntz,<sup>12</sup> Krogh,<sup>13</sup> Barcroft,<sup>14</sup> Henderson<sup>15</sup> and others showing that the O<sub>2</sub> and CO<sub>2</sub> contents of the blood are subject to considerable variations, particularly under operative conditions.

It is a commonplace laboratory exercise to clamp off the abdominal aorta below the diaphragm and witness the enormous rise in systemic pressure (carotid) which results. Simultaneous clamping of the inferior vena cava with the aorta will likewise produce the rise in systemic pressure. But if the two vessels are not clamped simultaneously, the mechanical effect will depend on the order in which the two are clamped. Clamping the inferior vena cava without clamping the aorta will produce a great fall in blood pressure for very obvious reasons. Hence, if the vena cava be clamped even as much as fifteen seconds before the aorta, the rise in carotid pressure is not so great as when the two are clamped together. *Vice versa* should the aorta be clamped first and even a small interval of time intervene before the vena cava is clamped, the blood from the abdominal viscera will continue to flow into the thorax until the *vis a tergo* is exhausted and the pressure on clamping the aorta will mount even higher, oftentimes so high as to cause heart failure.

These mechanical effects must, of necessity, affect the circula-

<sup>12</sup> Zuntz, N.: *Deutsch. med. Wochenschr.*, 1892, p. 109.

<sup>13</sup> Krogh: *Skand. Arch. f. Physiol.*, xxiii, p. 179 *et seq.*, 1910.

<sup>14</sup> Barcroft: *Ergeb. d. Physiol.*, vii, p. 699, 1908.

<sup>15</sup> Henderson: *Amer. Journ. of Physiol.*, xxi, p. 126, 1908; xxiv, p. 66, 1909.

tion through the lungs, the aëration of the blood in other words, and in turn the exchange of oxygen and carbon dioxide between blood and outside air.

Suppose the two vessels be clamped exactly at the same moment. If the heart remains competent to empty itself against the increased pressure, the blood will of necessity circulate more rapidly through the lungs, unless the heart compensates by beating more slowly. On the other hand, if the heart be not competent, it may go into fibrillations or beat imperfectly, in which case blood will accumulate in the lungs, producing passive congestion.

The former set of conditions should result in a decrease in the carbon dioxide in the blood because, the blood being exposed more often to the alveolar air, the carbon dioxide has more opportunity to escape. The latter set of conditions should result in an interference with oxygen absorption with or without a decrease in the carbon dioxide.

#### EXPERIMENTAL PART.

Reasoning along these lines, the writers have undertaken to determine to what extent clamping of the abdominal aorta and inferior vena cava would alter the carbon dioxide content of the blood as it leaves the heart (carotid artery).

#### *Method.*

The method of procedure in the earlier, orienting experiments was as follows. Normal dogs were anaesthetized with chloretone. Urethane, which Porges and Porges and Salomon employed, has been avoided because it has been the experience in this laboratory that this drug excites the respiratory center of dogs much more than does chloretone. Morphine has likewise been avoided, except in one experiment, because it tends to increase the  $\text{CO}_2$  in the blood.<sup>16</sup>

When anaesthesia was fully established, the abdominal incision was made, the abdominal aorta exposed just above the origin of the coeliac axis, and hemostatic clamps were adjusted all ready to be closed at a signal. Before clamping, the pulse and respiration were usually counted and the control sample of blood was

<sup>16</sup> Cushny: *Textbook of Pharmacology*, 1910, p. 221.

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drawn. By these precautions one had knowledge of the condition of the animal just before the crucial operation. Then at a signal the two clamps were closed simultaneously, the closure of the vessels being immediately verified by examination. After the lapse of a varying interval of time, during which the pulse and respiration were counted frequently, the second sample of blood for analysis was drawn in the same manner as the first.<sup>17</sup>

The blood analyses were made by the chemical method of Haldane<sup>18</sup> using the apparatus devised by Brodie.<sup>19</sup> All determinations were made in duplicate.

The results of the preliminary experiments on five animals are presented in Table I.<sup>20</sup> It is evident, from an examination of these results, that a very great change in the aëration of the blood is brought about by the occlusion of these vessels. How serious a matter this change is for the life of the animal is seen in the fact that Dog III survived for only twenty minutes after the obstruction was accomplished. In all probability death was due to failure of the left ventricle.

*Out of the four experiments in which a second analysis of blood was made, three exhibit a marked fall in the carbon dioxide of the arterial blood. Two show, in addition, a material fall in the oxygen content.*

It is not to be supposed that the blood alone loses carbon dioxide. Arterial blood, containing less than the usual percentage of CO<sub>2</sub>, will carry away CO<sub>2</sub> by diffusion and this will continue the more rapidly the more the tension in the tissues exceeds that of the

<sup>17</sup> The clamps which have been used for obstructing the vena cava are those known to surgeons as gastero-enterostomy clamps, fitted with rubber. Much difficulty has been experienced in placing a clamp on the aorta above the origin of the coeliac axis without serious rupture of the diaphragm and a number of animals were killed prematurely in this way. After this experience, however, it was found that by exposing the coeliac axis itself and just above it applying the clamp in such a way as to include the arcuate fibres about the aorta within the clamp it was possible to effect a complete obstruction without injury to other structures. Later a heavy wrapping cord was passed about the aorta at this point by means of a ligature carrier.

<sup>18</sup> Haldane: *Journ. of Physiol.*, xxii, p. 465, 1897-8; Haldane and Barcroft: *Ibid*, xxxii, p. 232, 1902.

<sup>19</sup> Brodie: *Ibid*, xxxix, p. 391, 1910.

<sup>20</sup> Cf. *Proc. Soc. Exp. Biol. and Med.*, x, p. 174, 1913.

arterial blood. The total amount of extra carbon dioxide appearing in the respiration after the clamps are applied, therefore, will depend upon the amount of the gas stored in the tissues. There is no perfectly satisfactory method of estimating the total carbon dioxide stored in the body at one time; hence it is impossible, from the percentages in the blood, to say just how much would escape in a given time. There is scarcely any doubt, however, that sufficient  $\text{CO}_2$  has escaped from the animal's body in each of the three experiments, to cause a considerable rise in the respiratory quotient had it been determined for the period during which the vessels were clamped.

#### *Respiration experiments.*

Proof of the correctness of this view could only be had by repetition of the experiments of Porges accompanied by blood-gas analyses. Should the carbon dioxide of the arterial blood fall as in the foregoing experiments, during a respiration period occurring immediately after clamping of the vessels, and showing a higher R. Q., the conclusion would be irresistible that the higher quotient was due simply to an alteration in the rate of discharge and not an alteration in the rate of production of this gas. Again if the factor of over-ventilation, in the sense of increased breathing, were a controlling one, a period of exaggerated breathing preceding the clamping off of the vessels should nullify the effect of clamping on the respiratory quotient. It was desired also to make blood-pressure determinations before and after clamping to ascertain if possible what change is produced in the rate of flow through the lungs.

#### *Method of respiration experiments.*

The apparatus used was a respiration incubator constructed for the special purpose of studying the respiratory metabolism in new-born infants. It consists of a copper chamber ( $30 \times 32 \times 76$  cm.) placed inside a Freas electric incubator,<sup>21</sup> by means of which the chamber can be kept at a constant temperature, and connected

<sup>21</sup> The Freas incubator can be purchased from Eimer and Amend, New York. The assemblage of apparatus as used in these experiments will be described in detail soon.

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to a small Benedict<sup>22</sup> respiration machine, by means of which it is ventilated.

The chamber will accommodate a dog of 6–10 kgm. The entire cubic contents of the air circuit is about 80 liters and, with the subject inside, the air space is correspondingly less. This fact permits of the determination of the R. Q. by the well-known method of Benedict<sup>23</sup> with an unusual degree of accuracy; for the reason that a small variation of temperature or of barometric pressure makes but a slight error in the oxygen determination. By making residual analyses at the beginning and end of each respiration period, even these errors may be eliminated. The apparatus has been thoroughly tested by burning alcohol inside it, with results, for the R. Q., very close to the theoretical value (0.666); namely, 0.661, 0.654, 0.662, 0.667.

For the blood-pressure readings the pulse-pressure instrument of Dr. C. J. Wiggers,<sup>24</sup> who very kindly instructed us in its use, was employed. Instead of the usual levers for graphic records, the maximal and minimal pressure tubes were connected directly to long mercury manometers and the maximum and minimum pressures were read off the millimeter scale directly. It was necessary to have unusually long manometers on account of the very great rise in pressure which often, though not always, takes place on obstructing the vessels. In one of the earlier experiments of this series the mercury was blown out by the excessive pressure and some 50 cc. of blood escaped from the carotid artery in the confusion which followed. The pressure readings were satisfactorily obtained in only two experiments. In order to obtain the true pulse pressure and not the maximo-minimal pressures<sup>25</sup> the readings were made while the respirations of the animal were inhibited temporarily by central stimulation of the vagus nerve.

All of the animals were anaesthetized with chloretone (usually given by stomach) and were tied lightly on an ordinary laboratory dog-board which had been sawed off to fit the respiration chamber. Once the dog is in the chamber and the latter sealed air-tight

<sup>22</sup> Benedict, F.G.: *Amer. Journ. of Physiol.*, xxiv, p. 345, 1909.

<sup>23</sup> U.S. Dept. of Agriculture, Office of Experiment Stations, Bulletin 175, 1907.

<sup>24</sup> Wiggers, C.J.: *Amer. Journ. of Physiol.*, xxx, p. 233, 1912.

<sup>25</sup> Wiggers, C.J.: *loc. cit.*



the respiration experiment begins (after a short preliminary period of 10 to 25 minutes during which the chamber is being ventilated by the Benedict machine) on the second of the minute, by throwing a switch and turning a valve excluding the absorbers. Oxygen is admitted automatically throughout the period, but at the end the pressure is brought to the starting-point pressure by hand. The period was usually one hour in length.

*I. Experiment on depancreatized dog showing higher R. Q.* The first subject of this series was a depancreatized dog (Table II). That the animal was thoroughly diabetic is seen by the R. Q. obtained in two successive hours at the beginning of the experiments. The blood sample drawn at 3.12 p.m., just three minutes before clamping the vessels, showed a rather low percentage of both gases. It is well known that in diabetic subjects the CO<sub>2</sub> tension falls at times to a very low point.<sup>26</sup> Another sample drawn fifteen minutes after clamping however shows both gases still farther reduced—the carbon dioxide more than 10 per cent. Twenty-five minutes after taking this sample of blood the second respiration experiment began and continued for nearly two hours. The R. Q.s are decidedly increased, although the total respiratory exchange is very much reduced. *Accompanying the higher R. Q. is a very great depression in the carbon-dioxide content of the blood.* In view of the elevated blood pressure, which continued for twelve minutes at least after clamping the vessels, and the increased percentage of oxygen it seems likely that the true explanation of the lower percentage of CO<sub>2</sub> and hence of the higher R. Q. in this case is an increased aëration of the blood by more rapid circulation through the lungs.

*II. Experiment in which the R. Q. remained the same after clamping the vessels.* This was a normal dog, anaesthetized as usual. Blood pressures were determined before the respiration experiment. The dog had been fed the day before on dog biscuit, containing a high percentage of carbohydrate, and may have eaten some of it left over from the previous day, on the morning of the experiment. The R. Q. is rather high (probably for this reason) the first hour, but fell the second hour to a point more nearly within the range of a true *nüchtern* value.

<sup>26</sup> Beddard, Pembrey and Spriggs: *Lancet*, 1903, I, p. 1366.

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Upon clamping, the blood gases fell rapidly within the next fifteen minutes and the blood pressures which were high at first fell rather suddenly to very low levels. *The R. Q. did not rise in the second respiration period and the CO<sub>2</sub> did not fall as in the previous experiment.* The explanation of the low blood pressure was found at autopsy in the fact that the clamp on the aorta had caught a bit of the stomach and for this reason was not quite competent to hold the arterial pressure. It was possible, after sectioning the aorta below the clamp, to squeeze blood through. The animal therefore must have bled into his abdominal vessels until the arterial pressure reached a level which could no longer pass the obstruction.

This experiment proves the relatively greater importance of CO<sub>2</sub> than of O<sub>2</sub> in the blood in determining the external R. Q. Oxygen is not stored in the tissues in any quantity as is carbon dioxide; consequently a considerable change in the O<sub>2</sub> content of the circulating medium does not affect the R. Q. materially.

*III. Experiments in which the R. Q. fell after clamping the vessels.* In the next experiment (Table IV) the dog exhibited a high R. Q. in the first period but instead of falling as is usual the further the time from feeding, it rose. Unfortunately the respiration rate was not recorded during these preliminary periods. It must be supposed however that there had been a considerable over-ventilation of the lungs and a consequent *Auspumpung* of CO<sub>2</sub>; *for upon clamping the vessels there was no fall, to speak of, in the CO<sub>2</sub> content of the blood within the first twenty minutes, and during the subsequent respiration period the CO<sub>2</sub> rose to 54.8 per cent while the oxygen fell.* Again the CO<sub>2</sub> proves to be the determining factor; for its rise in the blood denotes a very considerable storage in the tissues and it is the holding back of this CO<sub>2</sub> which causes the R. Q. to fall to the extremely low level of 0.61 in the second period. The respiration apparatus was thoroughly tested immediately after this experiment and proved to be absolutely correct, giving a R. Q. with the alcohol flame of 0.667.

The results of the preceding experiments are fully confirmed in the following one (Table V) which was more complete. The dog had had no food since the previous day. The respiration rate was recorded during the preliminary respiration periods and established the cause of the high R. Q.s unquestionably to be the

*Auspumpung of CO<sub>2</sub>.* The temperature of the respiration chamber during these periods was very close to the critical temperature at which dogs begin to pant. This fact together with a rather light state of anaesthesia probably accounts for the high rate of breathing. *The pumping out of CO<sub>2</sub> in this case was so complete that upon clamping the vessels there was no reduction of the CO<sub>2</sub> in the blood, but instead a slight rise.* In all probability this rise started from a still lower level the moment the dog was removed from the respiration chamber, for the respiratory rate fell at once to normal. *In the respiration experiment which followed clamping of the vessels the R. Q., instead of rising, fell to an abnormally low point.* The clamps were absolutely competent.

Severe congestion of the lungs with oedema was found at autopsy, a circumstance which explains the very low percentage of oxygen found at the end. The carbon dioxide was not so high, however, as in the previous experiment. The rate of respiration declined rapidly and the dog was near death when removed from the chamber.

One other experiment, not reported in detail, was performed on a normal fasting dog, in which the R. Q.s in the preliminary periods were 0.72 and 0.85, while after clamping it was 0.67. The same explanations probably apply.

Two other experiments on depancreatized dogs were attempted but both died upon clamping of the vessels. Porges and Salomon succeeded in obtaining respiratory periods after ligation of the vessels in only four depancreatized dogs out of fifteen. There are obvious reasons why the animals do not survive longer. The strain upon the heart is tremendous. In several dogs, both normal and depancreatized, of this series, the heart failed at once and could not be revived. Aside from this the very rapid fall in the CO<sub>2</sub> percentage, which cannot be entirely compensated for by reduced rate (see Dog V, Table I) must produce a profound effect on all the higher brain centers. When, added to this, we consider that the congestion of the lungs is such as to interfere with the absorption of oxygen, the wonder becomes that so many animals survive as long as they do.

*Alkalinity of the blood.*

Rolly<sup>27</sup> has established, by a new and much improved method, the fact that in dogs operated after the Porges procedure, the H-ion concentration of the blood is increased and the OH-ion concentration is diminished. This observation has been confirmed in a single examination of the blood reaction made in these experiments. From Dog IX, 20 cc. of carotid blood were drawn (10 cc. into each of two centrifuge tubes containing 0.5 cc. each of 0.1 per cent hirudin solution) before clamping the vessels and again just after drawing the last sample of blood for gas analysis. Ten cc. of the hirudin plasma titrated to the first pink color of phenolphthalein with  $\frac{N}{10}$  NaOH required for the first sample 4.2 cc. and for the second 7 cc. *The acidity*, in other words, *had nearly doubled*, and yet in spite of this change the CO<sub>2</sub> was held back coincidentally so as to reduce the R. Q. to 0.633! From this single observation the indications are that this greater acidity (H-ion concentration) cannot be the only cause of the extra elimination of CO<sub>2</sub>.

## DISCUSSION OF THE FACTOR OF EXAGGERATED BREATHING.

This series of experiments was undertaken in the full expectation of finding mechanical factors adequate to explain *any alteration* in the R. Q. which could result from sudden obstruction of the main vessels leading to and from the abdominal organs. One such factor, exaggerated breathing, unquestionably is; for in these experiments it has been shown (Dogs VIII and IX) that increased respiratory activity may keep the quotient far above normal for at least two hours. That over-ventilation (*exaggerated breathing*) was present in the experiments of Porges and of Porges and Salomon may be inferred, in the absence of direct data, from the expressed assumption of Porges that after fifteen minutes of exaggerated breathing no more CO<sub>2</sub> could be pumped out. Furthermore it is the experience of this laboratory that urethane, which Porges and Salomon used, always excites the respiratory center (in dogs) and that it cannot always be controlled with moderate doses of morphine. In a former series of experiments in which the respiration apparatus was attached directly to the

<sup>27</sup> Rolly: *Münch. med. Wochenschr.*, 1912, Nos. 22 and 23.

trachea<sup>28</sup> urethane was tried and was given up for this very reason. In the original experiments of Porges and of Porges and Salomon, the higher quotients are doubtless due in part to this form of over-ventilation.

#### DISCUSSION OF THE FACTOR OF BLOOD FLOW THROUGH THE LUNGS.

That some other factor than exaggerated breathing may account for a great reduction in the  $\text{CO}_2$  of the arterial blood and therefore for a rise in the respiratory quotient after clamping of the vessels, is seen from the experiments with Dogs I, II and V (Table I) and Dog VI (Table II). In none of these experiments was any increased breathing observed. The blood-pressure determination with Dog VI gave a clue which it was hoped would lead to definite conclusions on the matter of blood-flow when the pulse pressures were more accurately determined in the experiments with Dogs VII and IX. Unluckily the leak in Experiment VII invalidated the blood-pressure findings, as a criterion of blood-flow in that experiment; for the mean pressure changed. In Experiment IX however it may be seen that the minute volume of blood-flow through the heart, and therefore through the lungs has changed greatly after clamping and that this change is consistent with the change in blood gases.

According to the law of von Recklinghausen<sup>29</sup> the amplitude of the pulse wave (pulse pressure) at any given mean pressure is a measure of the systolic output, provided the distensibility of the arterial wall is constant. The product of the pulse-pressure by the pulse-frequency is then a measure of the minute-volume.

There is no reason to suppose that the distensibility coefficient *per se* of the arterial system is in any way altered by the clamping of the aorta and vena cava. Therefore if the mean pressure remains about the same the product of pulse-pressure into pulse-frequency would afford a criterion of the effect of the operation on the blood-flow.

Referring to Table V it is seen that the pulse pressure just before clamping is three and one-half times as great as just after clamping. The mean pressure has risen slightly but not sufficiently

<sup>28</sup> Murlin and Greer: *Amer. Journ. of Physiol.*, xxvii, p. xviii, 1911.

<sup>29</sup> v. Recklinghausen: *Arch. f. exp. Path. u. Pharm.*, lvi, p. 1, 1906.

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to offset the difference in pulse pressure.<sup>30</sup> The pulse frequency is considerably higher before the operation than after it. Hence the blood-flow through the lungs has been greatly reduced by the operation. The surprising thing is that such a change in the blood-flow should not have produced a greater effect on the exchange of gases.

Two facts then stand out with some significance in the matter of blood-flow. In Experiment VI where the  $\text{CO}_2$  in the blood fell rapidly after clamping of the vessels (while the  $\text{O}_2$  rose), and the R. Q. as a consequence rose, the pulse pressure was maintained. Since there is no reason to believe that the pulse rate suffered any diminution (see Experiments I-V), the minute volume after clamping was at least as great as before. In Experiment IX where the  $\text{CO}_2$  in the blood rose slightly (while the  $\text{O}_2$  fell) and the R. Q. as a consequence was falling (after the previous over-ventilation) the minute volume was distinctly less. These two facts are offered not as final proof but as evidence, consistent as far as it goes, that

$$^{30} \text{ v. Recklinghausen's formula is } A = \frac{R}{\left(\frac{dI}{dp}\right)_{\mu}} \times 1/k \text{ where } A \text{ is ampli-}$$

tude or pulse pressure,  $R$  is pulse volume, the expression  $\left(\frac{dI}{dp}\right)_{\mu}$  denotes distensibility of the arterial wall, at the mean pressure and  $k$  is a constant determined by viscosity, diameter of vessels, etc. The pulse volume  $R$  then would be expressed by the formula  $\frac{A (\text{distensibility})}{1/k}$ .

Making substitutions from Table V the pulse volume before clamping would be  $\frac{22 \times \text{distensibility at 54}}{1/k}$ ; after clamping it would be

$$\frac{6 \times \text{distensibility at 72}}{1/k}.$$

Supposing the distensibility and the value of  $k$  to be the same the pulse volume before clamping is more than three times the value after clamping. The minute volume would be found by multiplying the value of the pulse-volume, or systolic output, by the pulse-frequency. Taking 210 as pulse-frequency just before clamping and 180 just after, the minute volume proves to be less than one-third its former value. In all probability this difference is too great; the point is to show that distensibility or the value of  $k$  would have to change a great deal to offset the difference in pulse pressure observed.

the altered rate of blood-flow through the lungs is an important factor in determining the  $\text{CO}_2$  (and  $\text{O}_2$ ) content of the blood and therefore in explaining the altered respiratory exchange.

CONCLUSION.

Whether one or both of the factors discussed above are controlling, there can be no doubt as to the significance of the blood-gas analyses. In each instance the blood-gas changes are consistent with the mechanical explanation of the altered respiratory quotients after clamping the vessels. Where the R. Q. rose (Experiment VI) the  $\text{CO}_2$  of the blood fell; where the quotient remained stationary (Experiment VII), the  $\text{CO}_2$  did not change; and where the R. Q. fell (Experiments VIII and IX), the  $\text{CO}_2$  in the arterial blood rose. Clamping off the blood from the abdominal organs therefore does not alter the character of the metabolism, and the experiments of Porges and of Porges and Salomon have no bearing on the problem of the oxidation of sugar.

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TABLE I.

*Dog I. 8 kgm. March 22, 1913. Chloretone per rectum.*

TIME	EVENT	PULSE	RESPI- RATION	BLOOD ANALYSIS	
				O <sub>2</sub>	CO <sub>2</sub>
<i>p.m.</i>				<i>per cent</i>	<i>per cent</i>
3.20	4.3 cc. carotid blood drawn			15.43	43.6
3.25	Vessels clamped simultaneously				
3.30		96	36		
3.40		144	30		
3.45		138	30		
3.54	4.2 cc. carotid blood drawn			15.52	22.53
3.55	Clamps removed				
4.00		120	35		

*Dog II. 12 kgm. April 10, 1913. Chloretone intraperitoneally.*

2.15	4.4 cc. carotid blood drawn			17.16	38.35
2.17	Vessels clamped simultaneously	66	35		
2.20		120	24		
2.35		102	12		
2.40		102	30		
2.41	3.2 cc. carotid blood drawn			17.16	37.47
2.47	Clamps removed				

*Dog III. 7.5 kgm. April 12, 1913. Chloretone anaesthesia.*

2.08		108	30		
2.20		114	24		
2.30		114	24		
2.35		120	15		
2.36	4.5 cc. carotid blood drawn			18.85	42.01
2.40		120	24		
2.46	Vessels clamped simultaneously; heart stopped				
2.58			24		
2.59	Artificial respiration				
3.00		96	30		
3.06		120	54		
3.10	Dog died; clamps on only 20 minutes; cause of death not apparent				



TABLE I.—Continued.

*Dog IV. 9 kgm. April 19, 1913. Chloretone by stomach.*

TIME	EVENT	PULSE	RESPIRATION	BLOOD ANALYSIS	
				O <sub>2</sub>	CO <sub>2</sub>
<i>p.m.</i>				<i>per cent</i>	<i>per cent</i>
1.50		138	35		
2.25		120	72		
2.32	4.4 cc. carotid blood drawn	138	66	19.52	39.42
2.38		132	60		
2.40	Vessels clamped simultaneously				
2.43		104	96		
2.52		126	64		
3.02		120	78		
3.15		120	80		
3.25		120	72		
3.35		126	72		
3.43	4.35 cc. carotid blood drawn			17.09	24.28
3.44	Clamps removed				
3.45		120	60		

*Dog V. 10 kgm. May 10, 1913. Morphine subcutaneously.  
Chloretone by stomach.*

2.28		96	34		
3.15		120	32		
3.17	4.15 cc. carotid blood drawn			13.32	51.06
3.20	Vessels clamped simultaneously				
3.22		102	16		
3.27		120	16		
3.33		120	14		
3.43		120	14		
4.03		120	14		
4.13		120	18		
4.18		120	16		
4.22	4.3 cc. carotid blood drawn			11.60	34.16
4.23	Clamps removed	120	24		
4.29		108	24		
4.39		108	24		

TABLE II.  
*Day VI. 6.1 kym. Depancreatized July 7, 1913. Experiment July 9, 1913. Chloretone by stomach.*

TIME	EVENT	BLOOD PRESSURE		PULSE PER MIN.	RESP. PER MIN.	BLOOD ANALYSIS		RESP. CO <sub>2</sub>	METAB. O <sub>2</sub>	R. Q.	TEMP. OF RESPIR. APP.
		Min.	Max.			O <sub>2</sub>	CO <sub>2</sub>				
a.m.											
11.27-12.27	In respiration apparatus										
p.m.											
12.27-1.27	In respiration apparatus										
1.30-2.30	Operation and arrangement of blood pressure apparatus										
3.00		70	80								
3.12	5.6 cc. blood drawn	66	84								
3.15	Vessels clamped simultaneously					11.06	34.72				
3.16		148	172								
3.17		130	174								
3.20		80	92	106	24						
3.27		80	94	112	28						
3.30	4.33 cc. blood drawn					9.85	24.07				
3.35	Returned to respiration apparatus										
3.55-4.55	Respiration experiment							2.35	2.07	0.83	30.0
4.55-5.42	Respiration experiment							1.74	1.55	0.82	30.4
5.45	4.6 cc. blood drawn										
6.00	Autopsy; clamps competent; no congestion of lungs					13.70	12.98				

TABLE III.  
Dog VII. 9.5 kgm. Normal, July 29, 1913. Chloretone by stomach.

TIME	EVENT	PULSE	RESP.	BLOOD PRESSURE		BLOOD ANALYSIS		RESP. CO <sub>2</sub>	METAB. O <sub>2</sub>	R. Q.	TEMP. OF RESP. APP.
				Min.	Max.	O <sub>2</sub>	CO <sub>2</sub>				
a.m.						per cent per cent		grams	grams		deg. C.
10.20	Exposed carotid	150	22								
10.24	Tied left vagus										
10.24	Cut left vagus	140	16								
10.25											
10.26				116	134						
				112	132						
				116	126						
10.27	Left vagus stimulated centrally	126		108	124						
10.28	Left vagus stimulated centrally	126		94	110						
10.29	Left vagus stimulated centrally	132		86	110						
10.30	Placed in respiration apparatus										
10.55-11.40	Respiration experiment		18						4.630	3.897	32.6
11.40-12.25	Respiration experiment		28-40						4.537	4.069	32.9
p.m.											
12.27	Laparotomy										
12.31		142	50								
12.46	Left vagus stimulated centrally	150		80	94						
12.47	Left vagus stimulated centrally	132		80	96						
12.48	Left vagus stimulated centrally	108		76	94						
12.50	4.4 cc. blood from carotid						25.73	37.38			
1.00				64	74						
1.03	Vessels clamped simultaneously			120	146						

TABLE III—Continued.

[illegible]

TABLE IV.  
*Dog VIII. 5.5 kgm. Normal. July 26, 1913. Chloretone intraperitoneally.*

TIME	EVENT	PULSE PER MIN.	RESP. PER MIN.	CAROTID BLOOD ANALYSIS		RESP. CO <sub>2</sub>	EXP. O <sub>2</sub>	R. Q.	TEMP. OF RESP. APP.
				O <sub>2</sub>	CO <sub>2</sub>				
				per cent	per cent	grams	grams		deg. C.
10.35-11.35 a.m.	Respiration experiment					4.512	4.096	0.861	31.0
11.35-12.35 p.m.	Respiration experiment					4.859	3.756	0.941	31.1
12.40	Dog operated	132	48						
12.49		154	56						
1.05									
1.08	4.6 cc. blood drawn								
1.10	Vessels clamped simultaneously			23.22	44.31				
1.12									
1.27		140	68						
1.30		140	52						
1.33	4.4 cc. blood drawn			20.48	43.95				
1.55-2.55	Returned to apparatus								
2.55-3.50	Respiration experiment		64			1.280	1.314	0.709	30.0
3.55	Respiration experiment		58-24			0.88	1.05	0.61	29.6
4.00	3.8 cc. blood drawn			7.1	54.80				
	Dog died; autopsy showed clamps competent; slight congestion of lungs								

TABLE V.  
*Dog IX. 6.5 kgm. Normal. August 1, 1915. Chloretone by stomach.*

TIME	EVENT	PULSE PER MIN.	RESP. PER MIN.	BLOOD PRESSURE		BLOOD ANALYSIS		RESP. CO <sub>2</sub>	METAB. O <sub>2</sub>	R. Q.	TEMP. OF RESP. APP.
				Min.	Max.	O <sub>2</sub>	CO <sub>2</sub>	grams	grams		deg. C.
a.m.											
10.41		120	20								
10.51	Exposed carotid	140	28								
11.01		140	28								
11.10	Connection with blood pressure apparatus	152	24	50	60						
11.12	Right vagus cut	120		40	70						
11.17	Right vagus stimulated centrally	132		42	68						
11.18	Right vagus stimulated centrally	132		38	64						
11.20	Dog in respiration apparatus		32								
12.00											
p.m.											
12.03	First period started		36								
12.35			42								
12.58											
1.01	End of first period		68					5.701	4.325	0.96	33.4
1.07			76								
1.35			110								
1.45			114								
1.50											
2.03	End of second period							6.573	4.598	1.04	33.5
2.05	Dog out of respiration apparatus										
2.20		180	32								

2.24	Left vagus cut and stimulated	210	50	70				
2.28	Left vagus stimulated	220	42	62				
2.30	3.4 cc. carotid blood drawn		•		10.72	35.36		
2.31	20 cc. carotid blood drawn for alkalimetry test; 10 cc. titrated 4.2 cc. $\frac{N}{10}$ NaOH							
2.35	Vessels clamped simultaneously							
2.41	Left vagus stimulated centrally	180	70	76				
2.42	Left vagus stimulated centrally	180	70	76				
2.43			42					
2.46	Left vagus stimulated centrally	192	68	74	lost	37.27		
2.50	4.5 cc. carotid blood drawn							
2.52	Dog in respiration apparatus							
3.08	Third period begins							
3.15			28					
3.30			28					
3.40			16					
3.55			18					
3.58	Third respiration period ends							
4.02	Dog out of respiration apparatus							
4.03	4.5 cc. carotid blood drawn							
4.04	20 cc. carotid blood drawn or alkalimetry; 10 cc. plasma titrated 7.0 cc. $\frac{N}{10}$ NaOH							
4.10	Dog died. Autopsy showed oedema of both lower lobes, clamps entirely competent						1.899	2.181 0.633 31.9
					4.6	39.36		





## THE SEPARATION OF *d*-ALANINE AND *d*-VALINE.

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In the ester method of protein hydrolysis the esterifiable amino-acids are separated by distillation into two fractions, a higher boiling containing aspartic and glutaminic acids, phenyl alanine, and serine, and a lower boiling fraction containing proline, *l*-leucine, *d*-isoleucine, *d*-valine, *d*-alanine, and glycocoll. For several years we have been trying to devise methods to approximate as nearly as possible a quantitative separation or determination of the six amino-acids composing the latter mixture.

Proline, unlike the other members of this fraction, is very soluble in alcohol,<sup>1</sup> and is partially separated from them by alcoholic extraction. The extract, however, usually consists of about two-thirds proline and one-third of a mixture of other amino-acids which have gone with the proline into solution in the alcohol. Proline, however, contains no primary amino nitrogen, while all the nitrogen of the other acids of this ester fraction is in the form of primary amino groups. Therefore, a determination of the total and the primary amino nitrogen,<sup>2</sup> respectively, in the extract permit one to calculate accurately the amount of proline, which is indicated by that of the non-amino nitrogen.

The other five amino-acids can be distributed by fractional crystallization among subfractions the composition of which varies greatly according to the proportions in which the different acids are present. As glycocoll and alanine dissolve at room temperature in only four parts of water, while the other three, particularly

<sup>1</sup> Fischer: *Ber. d. deutsch. chem. Gesellsch.*, xxxix, p. 530, 1906.

<sup>2</sup> Van Slyke: Quantitative Determination of Proline obtained by the Ester Method in Protein Hydrolysis, this *Journal*, ix, p. 205, 1911; Quantitative Determination of Aliphatic Amino Groups, this *Journal*, ix, p. 185, 1911 and xii, p. 275, 1912.

the leucine and isoleucine, are much less soluble, one can usually obtain by crystallization the greater part of the mixture in two fractions, a comparatively insoluble one consisting of the leucine and isoleucine, together with much of the valine, and a very soluble fraction containing glycocoll and alanine. For the quantitative determination of the proportions in which leucine, isoleucine, and valine are present in the less soluble fraction we have already published methods which have been utilized with satisfactory results.<sup>3</sup> More recently we have described the separation of glycocoll from alanine in the more soluble subfraction by means of glycocoll picrate, which is difficultly soluble in cold water.<sup>4</sup>

Besides the leucine-isoleucine-valine and the glycocoll-alanine crystallized fractions, however, one usually obtains another, intermediate between these two, containing alanine and valine in such proportions that they cannot be separated by crystallization. This paper presents a method for the separation of the alanine and valine of this intermediate fraction. One can now determine all the six amino-acids from the lower boiling ester fraction with a fair degree of accuracy. This does not mean that they are completely regained in the amounts in which they are present in the proteins. Losses which prevent this still occur in the esterification and distillation of the esters. The uncertainties, however, which were formerly connected with the separation of these amino-acids after the distillation, are now reduced to comparatively small proportions.

We have determined the following data, on which is based the method for separating valine from alanine, and from glycocoll in case this also should occur in the intermediate fraction.

*Data on which the separation is based.*

*d*-Alanine in the presence of 10 per cent sulphuric acid is precipitated by phosphotungstic acid as a crystalline salt which contains approximately 14 parts of phosphotungstic acid to 1 of

<sup>3</sup> Levene and Van Slyke: this *Journal*, vi, p. 391, 1909. Abderhalden and Weil have recently isolated from nerve tissue a third leucine isomer. We did not find evidence of it in casein or edestin; but if it proves to be a general constituent of the proteins still further development of special methods for this fraction will be necessary. *Zeitschr. f. physiol. Chem.*, lxxxiv, p. 39, 1913.

<sup>4</sup> Levene and Van Slyke: this *Journal*, xii, p. 285, 1912.

alanine. At 0° about twenty-four hours are required for precipitation of the maximum amount of alanine. The presence in solution of about 20 grams of phosphotungstic acid (in excess of the amount precipitated with the alanine) per 100 cc. of solution is required to insure most complete precipitation. Under these conditions the amount of alanine left in solution at 0° in 100 cc. of mother liquor is 0.15 gram. The concentration of free phosphotungstic acid can be increased up to at least 70 grams per 100 cc. of solution without either increasing or diminishing to a significant extent the solubility of alanine phosphotungstate.

*d*-Valine has under the same conditions the much greater solubility of 1.2 grams per 100 cc. Valine phosphotungstate shows, under proper conditions, very little tendency to form mixed crystals with alanine phosphotungstate. In case a mixture of the two is obtained, one can readily separate them by recrystallization from a solution containing 10 per cent of sulphuric and 20 per cent of phosphotungstic acid.

The solubilities of the phosphotungstates of both alanine and valine are very dependent upon the concentration of sulphuric acid present.

Glycocoll is precipitated under the same conditions as *d*-alanine, only 0.2 gram of glycocoll remaining in 100 cc. of mother liquor.

Lead acetate, recently recommended by Benedict and Murlin<sup>5</sup> for the removal of phosphotungstic acid from solutions containing amino-acids, is the most satisfactory reagent which we have found for freeing both alanine and valine from sulphuric and phosphotungstic acids. The precipitation of phosphotungstic acid is quantitative, and the small amount of lead sulphate remaining dissolved in the filtrate is readily removed by addition of an equal volume of alcohol. Five per cent, and sometimes even more, of the amino-acid present are usually adsorbed by the heavy precipitate, but the loss is less than when barium hydrate is used, and the amino-acid regained after removing the excess lead as sulphide and concentrating the solution to dryness contains less than 1 per cent of ash.

Natural leucine is precipitated by concentrated solutions of phosphotungstic acid, the precipitate being redissolved by sufficient excess of the acid, as found by Levene and Beatty. Leucine

<sup>5</sup> *Proc. Soc. Exp. Biol. and Med.*, 1912.

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may interfere with the purification of alanine as the phosphotungstate, however, and should be removed, either by crystallization or by precipitation as the lead salt<sup>6</sup> before the separation described below is begun.

Dilute methyl and ethyl alcohol are unsuitable solvents for the recrystallization of valine when even a small proportion of alanine is present; because the relative solubilities of the two amino-acids in water are reversed in both alcohols, in which alanine is much less soluble than valine. This is the case to a less marked extent with acetone, and it is, therefore, better suited to throw valine out of water solution in the presence of alanine. If to 100 cc. of water at 20° one adds 200 cc. of 80 per cent acetone, the resulting solution will dissolve 3.2 grams of alanine and 3.4 of valine. The solubility relations are such that one can add 3, 4, 5, 6, or 7 volumes of 80 per cent acetone with nearly the same effect. A mixture of 100 cc. of water and 700 cc. of 80 per cent acetone dissolves 2.5 grams of alanine and 3.4 of valine. Consequently, as the results are within a wide range independent of the volume of solution added, 80 per cent acetone affords a convenient means for throwing valine out of water solution in the presence of small amounts of alanine.

Because of the fact that alanine is much less soluble than valine in ethyl and methyl alcohol, especially the latter, it was thought that valine could, perhaps, be extracted from a mixture of the two amino-acids by means of methyl alcohol. It was found, however, that it was impossible to extract all the valine without also dissolving a large proportion of the alanine.

### *Precipitation and purification of alanine as phosphotungstate.*

The mixture of valine and alanine should preferably contain not over 50 per cent of valine. If more is present, part can readily be removed by recrystallizing from water, in which valine is much less soluble than alanine.

It is advisable, because of the appreciable solubility of alanine phosphotungstate, to precipitate it from as small a volume of 10 per cent sulphuric acid as will hold the valine in solution. In order to obtain at once alanine phosphotungstate free from valine

<sup>6</sup> Levene and Van Slyke: this *Journal*, vi, p. 391, 1909.

the volume of solution must be as great as 100 cc. for each gram of valine present. If the alanine phosphotungstate is recrystallized, however, one need use but 30 to 40 cc. for each gram of valine, recrystallizing once from a similar volume of fresh solution. One thus completes the separation, using in all only 60 to 80 per cent of the volume of solution required when one does not recrystallize, and one is also somewhat more certain of the absolute purity of the alanine. The process which gives the most satisfactory separation is the following:

The mixture of alanine and valine is dissolved in a hot solution which contains 10 grams of sulphuric acid per 100 cc. The volume of this 10 per cent sulphuric acid used should be 30–40 cc. for each gram of valine which analysis of the mixture indicates can, as a maximum, be present. In the hot solution one further dissolves enough purified phosphotungstic acid to combine in the ratio of 14:1 with the maximum amount of alanine which previous analysis has indicated can be present in the mixture, and in addition leave 1 gram of excess phosphotungstic acid for every 5 cc. of the 10 per cent sulphuric acid used. The use of a greater excess of phosphotungstic acid does not interfere with the separation, but leaves one an unnecessarily large amount to remove at the end of the operation. The solution prepared as above directed is placed in a refrigerator at 0° and allowed to remain there for at least twenty-four hours.<sup>7</sup> In case the volume of the solution is large, time must be allowed for it to cool before beginning to count the period allowed for crystallization. The precipitate separates in large, transparent crystals, which form a solid layer about the walls and bottom of the flask. When sufficient time has been allowed for the separation, the supernatant solution is decanted off as completely as possible. The crystals are then redissolved by heating with a volume of 10 per cent sulphuric acid equal to that originally used. Phosphotungstic acid, in the ratio of 1 gram to each 4 or 5 cc. of 10 per cent sulphuric acid used, is then dissolved in the hot solution, and the alanine phosphotungstate is again allowed twenty-four hours at 0° to crystallize. The supernatant solution is again decanted, and the crystals are washed with suction with a small volume of an ice-cold solution containing 10 per cent of sulphuric and 20 per cent of phosphotungstic acid.

<sup>7</sup> If only an ordinary ice box, which usually gives a temperature of 8°, is available, the flask should be immersed in ice water.

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### *Determination and isolation of the precipitated alanine.*

The alanine phosphotungstate is at once dissolved in hot water, where it forms a solution that is usually somewhat turbid. It is diluted in a measuring flask to such a volume that 10 cc. contain from 50 to 100 mgms. of alanine, and aliquot parts are used for determination of the nitrogen present. The determination is most conveniently performed by the nitrous acid method for determination of amino nitrogen.<sup>8</sup> If the micro-apparatus (cf. p. 121) is used 2 cc. of solution are sufficient; with the larger apparatus one uses 10 cc. The determination can also be done according to Kjeldahl, although in this case it is necessary to draw air through the mixture, while it is digesting with sulphuric acid, in order to prevent the violent bumping which the precipitated tungstic acid causes.<sup>9</sup> It is preferable to base the calculation of the amount of alanine present on the nitrogen determination rather than on the substance actually isolated, because, when the phosphotungstic acid is removed with lead, the bulky precipitate of lead phosphotungstate adsorbs several per cent of the alanine present, and the amount actually recovered is only 90–95 per cent of that in solution before the removal of the mineral acids. To the amount of alanine calculated from the nitrogen determination one may add a solubility correction for the amount dissolved in the total volume of solution from which the alanine was precipitated and recrystallized. This amount is calculated on the basis of a solubility of 0.15 gram of alanine per 100 cc.

The remainder of the solution, after the portion for the analysis has been removed, is washed into a Jena beaker and heated to boiling. A 20 per cent solution of neutral lead acetate is added in portions until an excess is present, and can be detected, by means of the sulphuric acid test, in a drop removed from the surface of the solution in the beaker. The heavy precipitate of lead sulphate and phosphotungstate is filtered with suction and washed thoroughly with water. The filtrate is concentrated to a volume

<sup>8</sup> This *Journal*, ix, p. 185, 1911, and xii, p. 275, 1912. As there is so much mineral acid present, it is advisable to add, to the nitrous acid solution in the apparatus, enough 4 or 5  $\times$  NaOH to nearly neutralize the sulphuric acid, before the solution containing the latter is run in.

<sup>9</sup> Denis: this *Journal*, viii, p. 427.

of about 50 cc. for each gram of alanine present, and mixed with an equal volume of 95 per cent alcohol. This precipitates a small amount of lead sulphate which had remained, owing to its slight but appreciable solubility in water. The solution is allowed to stand on the water bath for an hour or more to complete the precipitation, the sulphate is filtered off, and the excess of lead in the filtrate is removed with hydrogen sulphide. The lead sulphide is washed with water through which  $H_2S$  has been bubbled, and the filtrate is concentrated, preferably in vacuum, to a small volume. It is then transferred to a Jena glass evaporating dish and the concentration continued on the water bath until all the visible liquid has been evaporated. The drying is completed in a vacuum desiccator over sulphuric acid and potassium hydrate. It is not advisable to try to drive off with heat the last traces of water and acetic acid, for this is likely to somewhat discolor the substance. The product, dried in vacuum, is perfectly colorless, nearly ash-free (if pure reagents have been used), and free from valine.

Besides the alanine isolated as above described, a small amount, left in solution when the alanine phosphotungstate was precipitated, is later obtained from the mother liquors of the valine.

In case the original valine-alanine mixture contained glycocoll, the latter will now be found with the alanine, from which it can be separated as the picrate, according to the method described by us.<sup>10</sup>

*Determination and isolation of the valine.*

The decanted filtrates and the washings from the alanine phosphotungstate are diluted to a definite volume and the amino nitrogen determined in an aliquot part, in the manner described for the alanine solution. A special blank determination to ascertain the correction for the reagents should be made, using as the control solution 10 per cent sulphuric acid instead of water, as the presence of so much mineral acid increases the correction. The phosphotungstic and sulphuric acids are removed with lead acetate, as in the isolation of alanine, and the valine solution, free from mineral acids and bases, is concentrated on the water bath until the valine begins to crystallize at the surface. Two or three

<sup>10</sup> Levene and Van Slyke: this *Journal*, xii, p. 285, 1912.

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volumes of 80 per cent acetone are then added, and the mixture is rinsed, using more 80 per cent acetone, into a flask. This is stoppered to prevent evaporation of the acetone, and allowed to stand over night while the valine crystallizes. The latter is filtered, washed with 80 per cent acetone, and thus obtained free from alanine in a yield of 80 to 85 per cent of the amount present.

The filtrate from the valine contains the small amount of alanine which escaped precipitation by phosphotungstic acid, and an amount, usually about equal, of valine, which remained in solution in the dilute acetone. The filtrate is concentrated to dryness, weighed, and the alanine and valine separated with phosphotungstic acid as before. This second crystallization makes the separation practically quantitative.

When refrigeration facilities do not enable one to keep the solutions at 0° during the entire period while the alanine is being precipitated, one can let the solutions stand over night at room temperature, and then place them in ice water for several hours, stirring them occasionally to complete the crystallization at 0°. The precipitation is nearly, though not quite, so complete as when the solution is kept at 0° for the entire period.

Working at room temperature entirely, one can precipitate at least 75 per cent of the alanine in purity, using one-half the volume of solutions given in the above directions.

### *Purity of reagents.*

Because of the large amounts of lead acetate and phosphotungstic acid used, both reagents must be pure or the amino-acids obtained after their use will be accompanied by ash. The lead acetate should leave no residue after precipitation of a solution with hydrogen sulphide and evaporation of the filtrate to dryness. We have had no difficulty in obtaining good lead acetate from the manufacturers. The phosphotungstic acid should leave no residue after precipitation with pure lead acetate and evaporation of the filtrate. We purify the commercial phosphotungstic acid by Winterstein's method. The acid is dissolved in a small amount of water, from which it is shaken out with ether. With the latter it forms an oily solution much heavier than water. The ether solution is washed several times with water, and the ether is driven off on the water bath. The product is not hygroscopic, and forms a colorless solution.



## EXPERIMENTAL.

*Analysis of materials.*

*d*-Alanine was obtained from hydrolyzed silk by the ester method. The glycocoll accompanying the alanine in the amino-acids obtained from the low boiling fraction of esters was removed with picric acid,<sup>11</sup> and the *d*-alanine was purified by recrystallization from dilute alcohol. It gave the following figures on analysis.

Substance, 0.1195 gram; CO<sub>2</sub>, 0.1764 gram; H<sub>2</sub>O, 0.0825 gram.

Substance, 0.0909 gram; nitrogen gas at 21°, 763 mm. (nitrous acid method), 25.20 cc.

Substance, 0.1817 gram; solution (containing 1.3 mols. HCl), 2.4910 grams; concentration, 7.29 per cent; sp. gr., 1.03; rotation in 2 dm. tube with yellow light from a spectroscope, +2.07° ± 0.01°.

Substance, 0.1422 gram; solution in 20 per cent HCl, 2.5810 grams; concentration, 5.51 per cent; sp. gr., 1.087 at 25°; rotation in 2 dm. tube, +1.64° ± 0.01°.

	Found:	Calculated for C <sub>3</sub> H <sub>7</sub> O <sub>2</sub> N:
C.....	40.25	40.41
H.....	7.72	7.92
N.....	15.74	15.73
[α] <sub>D</sub> with 1.3 mols. HCl....	+9.77°	+10.30° (Calculated for HCl salt.) <sup>12</sup>
[α] <sub>D</sub> with 1.3 mols. HCl....	+13.78°	Calculated for amino- acid.
[α] <sub>D</sub> with 20 per cent HCl...	+9.72°	Calculated for HCl salt.
[α] <sub>D</sub> with 20 per cent HCl...	+13.69°	Calculated for amino- acid.

From the above figures it is apparent that the *d*-alanine was analytically pure and as free from *dl*-alanine as one can usually prepare it from hydrolyzed protein. The rotation is, as stated by Emil Fischer, practically unaffected by the amount of excess hydrochloric acid present.

*d*-Valine was prepared from casein by esterification and the use of our lead method.<sup>13</sup> The preparation gave the following figures on analysis:

Substance, 0.1203 gram; CO<sub>2</sub>, 0.2260 gram; H<sub>2</sub>O, 0.1013 gram.

Substance, 0.1081 gram; nitrogen, 22.9 cc. at 25°, 762 mm. (nitrous acid method).

<sup>11</sup> Levene and Van Slyke: this *Journal*, xii, p. 285, 1912.

<sup>12</sup> E. Fischer: *Ber. d. deutsch. chem. Gesellsch.*, xxxix, p. 464.

<sup>13</sup> Levene and Van Slyke: this *Journal*, vi, p. 391, 1909.

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Substance, 0.1510 gram; solution in 20 per cent HCl, 2.6240 grams; sp. gr., 1.10; rotation in 2 dm. tube with yellow light,  $+3.28^\circ \pm 0.01^\circ$ .

	Found:	Calculated for $C_6H_{11}O_2N$ :
C.....	51.21	51.24
H.....	9.43	9.47
N.....	11.97	11.96
$[\alpha]_D^{20}$ .....	$+25.93^\circ$	$+28.80^\circ$

The valine was analytically pure. The rotation was lower than that obtained by Fischer for synthetic *d*-valine,<sup>14</sup> but is as high as one usually obtains in the natural product after acid hydrolysis. As the valine obtained by acid hydrolysis of proteins usually has a rotation of  $+24^\circ$  to  $+26^\circ$ , the use of the above material gives one more nearly the conditions actually met in hydrolysis work than would employment of the optically pure synthetic substance.

### *Composition of alanine phosphotungstate.*

Levene and Beatty found that alanine combines with phosphotungstic acid to form a crystalline salt.<sup>15</sup> We have prepared the salt as nearly pure as possible in order to determine its composition. Preliminary preparations showed that the ratio of alanine to phosphotungstic acid was approximately 1:14. We dissolved the two constituents in this ratio (0.5 gram of alanine and 7 grams of phosphotungstic acid) in 15 cc. of normal hydrochloric acid, and let the solution stand over night while the salt crystallized. The crystals were filtered on a clay plate and dried over solid potassium hydrate until the chloride reaction disappeared. The product was further dried in a vacuum at  $100^\circ$ . The proportion of alanine was then determined by estimation of the amino nitrogen with nitrous acid. The results were:

N.....	1.036 per cent.
Alanine.....	6.57 per cent.
PTA.....	93.33 per cent.
Ratio, alanine: PTA.....	=1:14.1

The salt forms with water of crystallization. The air-dried substance loses 3.8 per cent of its weight when dried in vacuum at  $100^\circ$ , and the anhydrous salt when exposed to air takes up a sim-

<sup>14</sup> Ber. d. deutsch. chem. Gesellsch., xxxix, p. 2320.

<sup>15</sup> Levene and Beatty: Zeitschr. f. physiol. Chem., xlvii, p. 149, 1906.

ilar weight of moisture. This corresponds to approximately 3 molecules of water for 1 of alanine, the ratio, 1 alanine: 3 H<sub>2</sub>O, requiring 3.99 per cent water.

*Solubility of d-alanine phosphotungstate in varying concentrations of sulphuric acid.*

Solutions each containing 0.250 gram of *d*-alanine, 5 grams of phosphotungstic acid, and varying amounts of sulphuric acid were made up to 10 cc. volume and left at 0° for forty-eight hours. The solutions were then decanted through dry filter papers into the 10 cc. burette of the aminometer (apparatus for determination of amino nitrogen) described in this *Journal*, xii, p. 275. The nitrogen in the measured volume of filtrate was determined by the nitrous acid method, and from the result the amount of alanine present in 100 cc. of filtrate was calculated. The percentages of sulphuric acid indicate grams per 100 cc. of solution.

TABLE I.

CONCENTRATION H <sub>2</sub> SO <sub>4</sub>	ALANINE IN 100 CC. OF FILTRATE
per cent	grams
3	0.56
4	0.38
5	0.36
6	0.30
8	0.19
10	0.14
10	0.15
12	0.16
14	0.18
16	0.18

As 0.250 gram of alanine combines with 3.5 grams of phosphotungstic acid, the excess of the latter in solution was 1.5 grams, or 15 grams per 100 cc. The above table indicates that, in the presence of this excess of phosphotungstic acid, sulphuric acid decreases the solubility of alanine phosphotungstate, the maximum effect of the sulphuric acid being exerted in 10 per cent concentration. Under these conditions the solubility of alanine at 0° is only 1 gram per 700 cc. of solution.

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*Effect of the concentration of free phosphotungstic acid on the solubility of d-alanine phosphotungstate in 10 per cent sulphuric acid at 0°.*

Portions of 50 mgm. of *d*-alanine were dissolved in 5 cc. each of 20 per cent sulphuric acid in test tubes, and varying amounts of a solution containing 2 grams of phosphotungstic acid per cubic centimeter were added to the different solutions, all of which were then made up to 10 cc. with water allowed to stand thirty hours at 0°. The amounts of alanine remaining in solution were then determined as described in the preceding section. The excess phosphotungstic acid was estimated by subtracting from the amount added the 0.7 gram combining with 0.05 gram of alanine.

TABLE II.

PTA ADDED PER 100 CC.	EXCESS PTA PRESENT PER 100 CC.	ALANINE IN 100 CC. OF FILTRATE
<i>grams</i>		<i>grams</i>
15	8	0.22
20	13	0.21
25	18	0.18
30	23	0.15
40	33	0.14
60	53	0.15
80	73	0.13

It is evident that about 20 per cent of free, excess phosphotungstic acid in solution insures a maximum precipitation of the alanine at 0°. At 20°, in the presence of 20 per cent phosphotungstic acid solution, the solubility is 0.3 gram per 100 cc.

*Time required for the precipitation of d-alanine phosphotungstate at 0°.*

Portions of 0.05 gram of *d*-alanine were dissolved with 3 grams of phosphotungstic acid in 10 cc. of 10 per cent sulphuric acid. The solutions were left at 0° for varying periods, at the end of which they were decanted through dry filters, as in the solubility determinations described in the preceding sections, and the nitrogen remaining in solution was determined.

TABLE III.

TIME ALLOWED FOR PRECIPITATION	ALANINE IN 100 CC. FILTRATE
<i>hours</i>	<i>grams</i>
3	0.21
6	0.20
15	0.17
22	0.16
40	0.14

While the greater part of the alanine is precipitated in three hours, over twenty are required for the complete attainment of solubility equilibrium.

*Solubility of dl-alanine in 10 per cent sulphuric acid containing varying concentrations of phosphotungstic acid.*

The results in the following table show that the phosphotungstate of *dl*-alanine is more than twice as soluble at 0° as that of *d*-alanine. The conditions of the solubility tests were the same as those of the foregoing experiment.

TABLE IV.

PTA ADDED PER 100 CC.	EXCESS PTA PRESENT PER 100 CC.	ALANINE IN 100 CC. FILTRATE
<i>grams</i>		<i>grams</i>
10	3	0.43
20	13	0.35
30	23	0.35
60	53	0.37
80	73	0.37

*Solubility of d-valine phosphotungstate in varying concentrations of sulphuric acid at 0°.*

Portions of 0.4 gram of valine were dissolved with 6 grams of phosphotungstic acid each in 5 cc. of 2, 4, 6, 8, 10, and 12 per cent sulphuric acid respectively. The amount of phosphotungstic acid was found by a separate experiment to be a sufficient excess to depress the solubility of the valine to its minimum. The solutions were cooled to 0° and kept at that temperature for three days. The solubilities of the valine were then determined as in

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the similar experiments with alanine. The solution with only 2 per cent of sulphuric acid showed no precipitate. The others showed crystalline precipitates varying in bulk with the concentration of the sulphuric acid. The percentages of sulphuric acid indicate grams per 100 cc.

TABLE V.

H <sub>2</sub> SO <sub>4</sub>	VALINE IN 100 CC. OF FILTRATE
per cent	grams
4	4.95
6	2.78
8	1.87
10	1.21
12	0.88

At 20° the solubility in 10 per cent sulphuric acid in the presence of an excess of phosphotungstic acid is 3.4 grams per 100 cc.

### *Solubility of valine and alanine in varying concentrations of acetone.*

As stated before, acetone was found a better agent than methyl or ethyl alcohol for throwing valine out of solution in the presence of the small proportions of alanine that escape precipitation with the main crop of alanine phosphotungstate. To ascertain the optimum proportion of acetone to add to the water solution of valine in order to cause it to crystallize most completely without carrying down alanine also, the solubilities of the two amino-acids in varying concentrations of acetone were determined at 20°. Fifteen cubic centimeters of the solvent were in each case shaken two hours with an excess of amino-acid, and 10 cc. of the filtered solution evaporated in a weighed dish.

TABLE VI.

ACETONE	VALINE SOLUBLE IN 100 CC. AT 20°	ALANINE SOLUBLE IN 100 CC. AT 20°
per cent	grams	grams
100	0.008	0.002
90	0.028	0.012
80	0.164	0.097
66.7	0.560	0.402
50	1.290	1.315

The following table shows that when 80 per cent acetone, in the ratio of from 2 to 7 volumes, is added to 1 volume of water, the solvent power of the water for alanine and valine is reduced to a point which remains nearly the same, whether 2, 3, 4, 5, 6, or 7 volumes of the 80 per cent acetone are added. The decrease in solubility caused by increasing the percentage of acetone is approximately compensated by the increase in volume.

TABLE VII.

80 PER CENT ACETONE ADDED TO 100 cc. OF WATER	ACETONE IN THE MIXTURE	SOLUBILITY IN 100 cc. OF THE MIXTURE		AMINO-ACID DISSOLVED IN THE TOTAL VOLUME OF MIXTURE	
		Alanine	Valine	Alanine	Valine
cc.	per cent	grams	grams	grams	grams
200	53.3	1.08	1.16	3.24	3.48
300	60.0	0.71	0.85	2.84	3.40
400	64.0	0.52	0.67	2.60	3.35
500	66.7	0.40	0.56	2.40	3.36
600	68.6	0.35	0.48	2.45	3.36
700	70.0	0.31	0.43	2.48	3.44

The solubilities in the third column were graphically interpolated from those given in the preceding table.

*Separation of a mixture of d-valine and d-alanine.*

The following separation serves as an example of the application of the method.

One gram each of *d*-valine and *d*-alanine was dissolved in 35 cc. of hot 10 per cent sulphuric acid (prepared by diluting 10 grams of acid to 100 cc.) with 23 grams of purified phosphotungstic acid. The solution was allowed to stand till it had cooled to room temperature, and was then placed in a refrigerator at 0° for twenty-four hours. The crystals which had separated formed a solid layer about the walls and bottom of the flask. The supernatant liquid was decanted off, and the crystals were redissolved on the water bath with 35 cc. of fresh 10 per cent sulphuric acid. Eight grams of phosphotungstic acid were then dissolved in the hot solution, which was cooled and placed in the refrigerator for twenty-four hours as before. The mother liquors were again decanted off, and the crystals were quickly washed on a suction funnel with

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several small portions of a solution containing 10 grams of sulphuric acid and 20 grams of phosphotungstic per 100 cc., the washing solution being at a temperature of 0°.

*Alanine.* The crystals were transferred as completely as possible with a spatula from the funnel to a Jena beaker. A small residue adhering to the funnel and filter paper was washed into the beaker with hot water, and the flask in which the crystals had formed was also washed out with hot water, in order to obtain a few crystals of alanine phosphotungstate which the previous washing had not removed to the funnel. Enough water was added to the alanine phosphotungstate to bring the volume to 75-100 cc., and the beaker was covered and heated on the water bath until the crystals were dissolved to a slightly turbid solution. The latter was transferred to a 150 cc. measuring flask and diluted to the mark. Two cubic centimeters of the solution used for determination of amino nitrogen in the micro-apparatus gave 3.37 cc. of nitrogen gas at 25°, 758 mm., indicating 0.1398 gram of nitrogen, or 0.889 gram of alanine in the entire solution. The remaining 148 cc. of solution were treated as described on pp. 108 and 109 to remove phosphotungstic and sulphuric acids. The alanine regained weighed 0.83 gram, and gave the following figures on analysis.

Substance, 0.1222 gram; ash, 0.0013 gram; substance, ash-free, 0.1209 gram; CO<sub>2</sub>, 0.1777 gram; H<sub>2</sub>O, 0.0855 gram.

*Rotation in 20 per cent HCl:* Substance, 0.1029 gram = 0.1016 ash-free; solution, 1.9060 grams; concentration, 5.33 per cent; sp. gr., 1.1; rotation in 1 dm. tube, +0.80°.

	Found:	Calculated for <i>d</i> -alanine:
C.....	40.10	40.40
H.....	7.92	7.92
$[\alpha]_D^{20}$ .....	+13.7° ±0.2°	+13.7°

It is evident that the precipitate consisted of pure alanine phosphotungstate. The correction for the solubility of alanine as phosphotungstate in 70 cc. of solution under the conditions of precipitation and recrystallization is  $0.70 \times 0.15 = 0.105$  gram of alanine. Adding this to the 0.889 gram precipitated gives 0.994 gram of alanine found to be present out of the 1 gram originally added.

*Valine.* The filtrate and washings from the alanine phosphotungstate were diluted to 150 cc. and 2 cc. of the solution taken



for determination of amino nitrogen. The nitrogen obtained measured 3.28 cc. at 25°, 758 mm., indicating 0.1362 gram of nitrogen in the entire solution. This is equivalent to 1.001 gram valine besides the 0.105 gram of alanine which, according to the solubility of alanine phosphotungstate, should be present. The remaining 148 cc. of solution were freed from sulphuric and phosphotungstic acids with lead acetate and concentrated, first in vacuum, then in a Jena glass dish on the water bath, until valine began to crystallize at the surface. About 3 volumes of 80 per cent acetone were stirred into the hot solution, which was then transferred, with the aid of more 80 per cent acetone, to an Erlenmeyer flask. The flask was stoppered and the valine allowed to crystallize in the ice box. The crystals, washed with 80 per cent acetone, weighed 0.78 gram, and gave the following analytical figures.

*Analysis:* Substance, 0.1198 gram (no ash); CO<sub>2</sub>, 0.2258 gram; H<sub>2</sub>O, 0.1108 gram.

*Rotation in 20 per cent HCl:* Substance, 0.0811 gram; solution, 1.291 grams; concentration, 6.28 per cent; sp. gr., 1.1; rotation in 1 dm. tube, +1.78° ± 0.01°.

	Found:	Calculated for d-valine:
C.....	51.38	51.24
H.....	9.41	9.47
[α] <sub>D</sub> <sup>20°</sup> .....	+25.8°	+25.9°

The filtrate from the above crop of valine was concentrated to dryness, taken up with 25 cc. of 10 per cent sulphuric acid, and the alanine precipitated with 6.5 grams of phosphotungstic acid. The precipitate was dissolved in hot water and the solution diluted to 50 cc. Two cubic centimeters gave 0.52 cc. of nitrogen, equivalent to 0.05 gram of alanine in the entire solution. This precipitation could have been made a little more complete if it had been performed in the same manner as the first, using only 7 or 8 cc. of solution instead of 25, and recrystallizing once. The filtrate from the alanine phosphotungstate was also brought to 50 cc. and 2 cc. taken for a determination of amino nitrogen, which yielded 1.89 cc. of gas at 24°, 766 mm., equivalent to 0.0266 gram of nitrogen in the entire solution. This indicates, besides the 0.04 gram of alanine soluble in the 25 cc. of solution from which it was precipitated, 0.17 gram of valine. When the solution had

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been freed from mineral acids and the product crystallized from dilute acetone, 0.13 gram of analytically pure valine was obtained.

*Analysis:* Substance, 0.1040 gram; ash, 0.0011 gram; substance, ash-free, 0.1029 gram; CO<sub>2</sub>, 0.1935 gram; H<sub>2</sub>O, 0.0896 gram.

	Found:	Calculated for C <sub>6</sub> H <sub>11</sub> O <sub>2</sub> N:
C.....	51.28	51.24
H.....	9.74	9.47

The total amount of analytically pure valine regained was 0.91 gram, or, making allowance for the portions of solution removed for nitrogen determination, 0.93 gram. The amount present, as calculated from the nitrogen content of the filtrate from the alanine, was 1.001 grams. The loss of 0.07 gram in isolation is due partly to loss in crystallization, partly to adsorption by the heavy lead precipitates formed when the mineral acids are removed, these precipitates always adsorbing a few per cent of the amino-acid present.

### CONCLUSION.

*d*-Alanine combines with phosphotungstic acid in the ratio of approximately 1:14 by weight, forming a crystalline salt. At 0°, in a solution containing, per 100 cc., 20 grams or more of phosphotungstic acid in excess of the amount combining with the alanine, and 10 grams of sulphuric acid, the solubility of alanine is only 0.15 gram per 100 cc. The solubility of *d*-valine under the same conditions is 1.21 grams per 100 cc. By alternate crystallization of valine as the free amino-acid and of alanine as the phosphotungstate, one can effect a practically quantitative separation of a mixture of the two amino-acids.

## THE GASOMETRIC DETERMINATION OF ALIPHATIC AMINO NITROGEN IN MINUTE QUANTITIES.

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In a previous number of this *Journal*<sup>1</sup> we have described an improved apparatus for the determination of amino nitrogen by the nitrous acid reaction. Its chief advantages over the form originally described<sup>2</sup> by us lay in its ability to be used an indefinite number of times without separating any of the parts, and in the fact that it permitted all the shaking to be done by a motor. By merely reducing its size this form of apparatus can be given an accuracy which brings the determination within the class of micro-methods. The gas burette of the micro-apparatus holds 10 cc. The upper part, measuring the first 2 cc., is of only 4 mm. diameter, and is divided into  $\frac{1}{50}$  cc. divisions. The remainder is wider, and is divided into twentieths. In order to keep the correction necessary for the reagents small, it is preferable that the amounts of the latter should be reduced in proportion to the volume of nitrogen obtained for measurement. The deaminizing bulb is, therefore, of only 11 to 12 cc. content, and the 10 cc. burette on the larger apparatus is replaced by one of 2 cc. capacity. Only 10

<sup>1</sup> This *Journal*, xii, p. 275, 1912.

<sup>2</sup> *Ibid.*, ix, p. 185, 1911. The apparatus is designed only for use with a motor. It can be obtained from Emil Greiner, 45 Cliff Street, New York, with motor for either direct or alternating street current, or from Robert Goetze, Leipzig.

The substance in the nitrite which gives the small amount of gas obtained on blank determinations we have never been able to identify or remove. As a matter of fact, while some brands of commercial grades of nitrite are entirely unsuitable, others give results as good as those obtained with the most high priced "reagent" or "zur Analyse" preparations. For the last two years we have used the ordinary grade supplied by the Powers-Weightman-Rosengarten Company with uniformly good results.

cc. of nitrite solution and 2.5 cc. of acetic acid are required for an analysis, and the correction for the reagents is 0.06 to 0.12 cc., according to the quality of the nitrite employed. The same size of modified Hempel pipette (see previous article) can be used for the small as for the large apparatus, and, because of the small amounts of nitric oxide absorbed, it lasts for an almost indefinite number of analyses without change of the permanganate solution. With the micro-apparatus the error need not be more than 0.005 mgm. of nitrogen when 2 cc. or less of gas are measured, or 0.01 mgm. when more is obtained. Consequently one can analyze one-fifth the amount of substance required for the larger apparatus without reducing the percentage accuracy.

The advantages of the micro-apparatus are: (1) It requires only 0.5 mgm. of amino nitrogen for an analysis accurate to within 1 per cent. (2) It uses up relatively small amounts of reagents. (3) Having shorter dimensions and being of equally thick glass, it is relatively stronger than the larger apparatus. In point of rapidity the little apparatus has, if anything, a slight advantage over the large one. On a warm day we have made as many as ten accurate analyses per hour with the former. The minimum time required for the quantitative evolution of the nitrogen of  $\alpha$ -amino-acids in the thoroughly shaken apparatus is, at 15° to 20°, five to four minutes; at 20° to 25°, three minutes; at 25° to 30°, two and a half to two minutes. Because of its conveniences, we now use for physiological work the smaller apparatus almost exclusively.

Practically the only alteration from the mode of operation, already detailed in the previous description of the larger apparatus, is in the speeds at which the deaminizing bulb and the Hempel pipette are shaken. During the first stage of the analysis<sup>3</sup> the deaminizing bulb should be shaken by the motor at very high rate of speed, about as fast as the eye can follow, or an unnecessary amount of time is lost in freeing the apparatus from air. This stage is also much accelerated by warming the nitrite solution to 30° before it is used, in case a low room temperature has reduced the temperature of the solutions below 20°. In the third stage<sup>4</sup> when the nitric oxide is being absorbed by the permanganate, the

<sup>3</sup> This *Journal*, xii, p. 279, 1912.

<sup>4</sup> *Ibid.*, p. 280.

Hempel pipette should be shaken not faster than twice per second. Absorption is approximately as fast as when more vigorous shaking is used, and the latter is likely to break off from the residual gas small bubbles, which stick under the nearly horizontal upper side of the pipette and escape being drawn back into the gas burette for measurement.

Because of the small amount of nitrogen to be measured, it is especially necessary that in the first stage the removal of the air should be complete. This is assured by shaking the solution in the deaminizing bulb back each time, in this stage, until the bulb is two-thirds filled with nitric oxide.

One point in setting up the apparatus appears to require especial emphasis. The hook or wire loop from which the deaminizing bulb is suspended<sup>5</sup> should be perfectly rigid and hold the capillary outlet tube tightly. Otherwise the rapid shaking which is advantageous becomes, instead of a smooth vibration, a rattle, disagreeable to the operator and dangerous to the apparatus. Binding the tube to the holder with a strip of rubber band is a satisfactory method of insuring a firmly held apparatus.

The entire apparatus can be cleaned most conveniently by filling the burettes and deaminizing bulb with dichromate-sulphuric acid mixture. When the apparatus is in daily use it is a good practice to let it stand regularly over night filled with the cleaning mixture.

Two points in which every apparatus should be tested, as soon as it is set up, are the accuracy of the burettes and the tightness of the stopcocks. The two burettes are calibrated by weighing the water which they deliver; and the cocks are tested for their ability to remain air-tight when subjected to the suction or pressure of a column of water a meter high.

For most work, the solutions for analysis can be measured off with sufficient accuracy in the 2 cc. burette on the side of the deaminizing vessel. When especially accurate results are desired, however, one uses an Ostwald pipette, calibrated to deliver 1 or 2 cc. within 0.001 or 0.002 cc. respectively, and washes the burette twice, with six or seven drops of water distributed about the entire inner walls of the burette for each washing.

<sup>5</sup> See photograph, this *Journal*, xii, p. 277.

## Determination of Amino Nitrogen

The following results were obtained on four successive analyses from solutions measured in this manner, and illustrate fairly the accuracy which one can attain with the method. For each analysis 2 cc. of a 1 per cent solution of Kahlbaum's leucine were used, the amount of leucine being therefore 20 mgms.

NO.	N GAS	TEMPERATURE	PRESSURE	N EVOLVED	CALCULATED	ERROR
	cc.	deg. C.	mm.	mgms.	mgms.	mgms.
1	3.75	20	762	2.140	2.138	+0.002
2	3.74	20	762	2.133	2.138	-0.005
3	3.74	20	762	2.133	2.138	-0.005
4	3.77	21	762	2.141	2.138	+0.003

The following results, obtained with solutions measured from the 2-cc. burette, indicate that, when the latter is clean and the delivery careful, it gives nearly as consistent results as a pipette. For each analysis 2 cc. of a  $\frac{N}{10}$  solution of alanine were taken. The time allowed for the reaction was two and a half minutes.

NO.	N GAS	TEMPERATURE	PRESSURE	N EVOLVED	CALCULATED	ERROR
	cc.	deg. C.	mm.	mgms.	mgms.	mgms.
1	2.56	28	760	1.398	1.401	-0.003
2	2.58	28	760	1.409	1.401	+0.008
3	2.57	28	760	1.403	1.401	+0.002
4	2.57	28	760	1.403	1.401	+0.002

## IMPROVED METHODS IN THE GASOMETRIC DETERMINATION OF FREE AND CONJUGATED AMINO-ACID NITROGEN IN THE URINE.

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### *Total amino-acid nitrogen.*

Our original method for the determination of the total amino-acid nitrogen<sup>1</sup> (free plus conjugated), although it gave accurate results and has been used successfully by ourselves and others, required a somewhat cumbersome manipulation before the urines were ready for the final amino determination. After being acidified and heated in the autoclave to hydrolyze the urea, the ammonia was boiled off on a hot plate with lime, a process requiring careful watching for an hour or more, and rather offensive because of the odors evolved. The calcium sulphate and hydrate were then filtered, the filtration and washing requiring another hour. The washings, of about 500 cc. volume, were then concentrated on the water bath, which required two or more hours additional.

These manipulations have been greatly simplified by the ascertainment of the fact that one has merely to filter off the alkaline solution obtained after adding the lime, and concentrate the filtrate on the water bath to dryness, in order to drive off every trace of ammonia. This process dispenses entirely with the troublesome boiling off of the ammonia. The washing of the precipitate of calcium salts can also be avoided to advantage by making the mixture up to a definite volume before filtering, and taking an aliquot portion of the filtrate for the rest of the determination. All the operations are furthermore rendered more convenient by the use of the micro-apparatus for determining the

<sup>1</sup> Levene and Van Slyke: *This Journal*, xii, p. 301, 1912.

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amino nitrogen, which permits one to work with relatively small volumes of liquid, and yet have sufficient material for duplicates.

The present method is the following: 25 cc. of urine are mixed with 1 cc. of concentrated sulphuric acid and heated in an autoclave at 180° (oil bath temperature) for one and a half hours. The solution is then transferred to a 50-cc. flask and 2 grams of powdered calcium hydrate are added. The mixture is thoroughly shaken, made up to 50 cc., and filtered through a dry folded filter. Twenty cubic centimeters of the filtrate are measured into a Jena glass evaporating dish and concentrated to dryness on the water bath, the process of concentration requiring about a half hour. The residue is moistened with 1 cc. of 50 per cent acetic acid to bring the calcium hydrate and carbonate into solution, and is then washed into a 10 cc. flask and filled up to the mark. One can either use the entire solution for determination of the amino nitrogen in the large amino apparatus, or use 2-cc. portions for the micro-apparatus.

The length of time which the nitrous acid solution should be shaken in order to drive off all the amino nitrogen depends somewhat on the temperature. When the latter is 15–20° the time should be five to four minutes; for 20–25° it is three minutes; for 25–30°, two and a half to two minutes. It is preferable that the solution should be shaken vigorously with a motor and the time kept down to these limits, for the sake not only of rapidity but of accuracy. The reason for this is, that, even after removal of the ammonia and urea, urines contain small amounts of substances which belong to the class of *slowly* reacting amines, and are therefore not  $\alpha$ -amino-acids. The correction for this nitrogen can be ascertained in the same manner as the urea correction in amino determination on the blood,<sup>2</sup> by continuing the reaction, after the gas from the amino-acids has all been driven off, for a length of time equal to that utilized in decomposing the amino-acids (two to five minutes, according to the temperature), and then measuring the nitrogen that has been evolved during this second reaction-period. The correction is so small and constant, however, amounting to 0.2–0.3 per cent of the urine nitrogen, that it will for most work be found unnecessary to take it into account.

<sup>2</sup> Van Slyke and Meyer: *This Journal*, xii, p. 402, 1912.



The following results were obtained with normal human urines. For the final determination, 2 cc. of solution, equivalent to 2 cc. of urine, were used in the micro-apparatus. The temperature was 25°, the pressure 758 mm. for all measurements.

TABLE I.  
*Total amino nitrogen (free and conjugated).*

NO.	TOTAL N PER 100 CC.	N GAS	CORRECTED FOR AMINES OTHER THAN AMINO-ACIDS	AMINO NITROGEN PER 100 CC. URINE		PER CENT OF TOTAL N IN FORM OF AMINO-ACID NH <sub>2</sub>	
				Uncor- rected	Corrected (Amino- acids only)	Uncor- rected	Corrected
	<i>grams</i>	<i>cc.</i>		<i>mgms.</i>	<i>mgms.</i>		
1	1.211	1.15	1.05	31.1	28.4	2.57	2.35
		1.18	1.04	31.9	28.1	2.63	2.32
2	1.750	1.38	1.24	37.5	33.7	2.14	1.93
		1.30	1.26	37.7	34.3	2.15	1.96
3	0.833	1.19	1.08	32.4	29.3	3.89	3.52
		1.19	1.06	32.4	29.8	3.89	3.46
4	1.747	1.32	1.15	36.0	31.4	2.06	1.80
		1.30	1.15	35.3	31.4	2.02	1.80
5	1.309	1.10	0.97	31.0	27.0	2.37	2.09
		1.10	0.95	31.0	26.5	2.37	2.04

*Free amino-acid nitrogen.*

At the time our first paper was published we had been unable to find an agent which would remove or destroy the urea without either hydrolyzing conjugated amino-acids (hippuric acid, peptone, etc.) or removing free ones. Treatment in an autoclave, as described in the first part of this paper, efficiently destroys the urea, but it also hydrolyzes the conjugated amino-acids. Mercuric acetate with alkali precipitates urea completely, but it also precipitates almost all of the amino-acids. We were therefore forced to take advantage of the fact that urea reacts to the extent of only about 3 per cent with nitrous acid in the time that amino-acids react with 100 per cent of their nitrogen. After the amino-acid nitrogen has been driven off and measured one can ascertain

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the rate at which urea is evolving nitrogen in the same mixture, and thus make a correction for the small percentage of urea nitrogen decomposed while the amino-acids were finishing the reaction. The method is satisfactory when, as in normal blood, the excess of urea is not too great, but in the urine the urea nitrogen is about 100 times the normal free amino-acid nitrogen. For this reason the method could not be depended upon to give results more accurate than  $\pm 0.5$  per cent of the total nitrogen of the urine, and was therefore of value to determine amino-acids only when they were present in abnormally large amounts.

Recently, however, Marshall<sup>2</sup> has found in the urease of the soy bean the specific reagent for the destruction of urea. He shows that the water extract of the beans (prepared by extracting the pulverized beans with 10 parts of water for an hour at room temperature, then warming the mixture to 35°, adding one-tenth volume of  $\frac{N}{16}$  HCl to coagulate proteins, and filtering) completely hydrolyzes urea in the space of a few hours at 35° to ammonium carbonate. We have been able to confirm his results, and find furthermore that the extract under the conditions used does not appreciably hydrolyze hippuric acid, casein, or peptone, nor deaminate amino-acids.

One peculiarity which we have noticed is that the enzyme does not appear to follow the law of mass action. A given amount is required to decompose a urine under given conditions, and the dilution of the reacting substances can be varied greatly without much affecting results. It is essential, therefore, that the amount of extract taken should be sufficient to completely decompose all the urea present. This is most certainly assured by testing measured portions of the extract with urines of concentration at least as great as that of those to be analyzed, and ascertaining the proportion of enzyme necessary to give a maximum amount of ammonia. Our method is to take 3-cc. portions of urine in 100 cc. test tubes, add 1.0-, 1.5-, 2.0-, 2.5-, and 3.0-cc. portions of extract with a few drops of toluol to the respective tubes of the series, and place in a bath at 35° for three hours, or sixteen to twenty hours at room temperature. To each tube 2 cc. of saturated potassium carbonate solution are then added, and the

<sup>2</sup> Marshall: *This Journal*, xiv, p. 283, 1913.

ammonia is driven into 25 cc. of  $\frac{N}{10}$  hydrochloric acid by ten minutes' aeration according to Folin's recent method.<sup>4</sup> As an example the amounts of  $\frac{N}{10}$  acid neutralized in one such test were 17.85, 19.50, 20.20, 20.05, and 20.20 cc. respectively. Under the conditions, 3 cc. of urine required 2 cc. of enzyme solution.

The soy bean extract was tested for proteolytic activity in the following experiment. The extract added to 2 per cent urea solution in the proportions of 2 volumes of extract to 5 of urea solution, completely decomposed the latter in sixteen hours at room temperature. To test the action on a protein the following solution was prepared: 25 cc.  $H_2O$ ; 10 cc. soy bean extract; 0.125 gram casein; 1.0 cc. 0.1  $N$   $NaOH$  (to neutralize the casein); 0.2 gram  $NaCl$ .

In this and subsequent experiments toluene was used as preservative. The solution was alkaline to alizarine, acid to litmus. Two-cubic-centimeter portions, taken at once and after the solution had stood sixteen hours at  $25^\circ$ , were analyzed in the micro-apparatus for amino nitrogen. The time of reaction was four minutes in each case. The results were:

At once.....	0.26 cc. $N_2$ at $21^\circ$ , 767 mm.
After sixteen hours.....	0.29 cc. $N_2$ at $19^\circ$ , 767 mm.

A control performed on a solution without casein, but otherwise like the above, gave 0.20 cc. nitrogen gas under the same conditions. The free amino nitrogen in the amount of casein present in the first solution (5.5 per cent of the total nitrogen, see later paper by Van Slyke and Birchard) would yield 0.09 cc. of nitrogen gas if given time to react completely. It is evident from the above that, under conditions that result in complete decomposition of urea, casein is not appreciably hydrolyzed by the urease. In fact a solution of sodium caseinate without enzyme showed under the same conditions as the result of autohydrolysis, more increase in amino nitrogen (0.05 cc.) than that noted above.

In order to test the extract for the presence of an erepsin, the following solution was prepared: 25 cc.  $H_2O$ ; 10 cc. soy bean extract; 0.200 gram Siegfried's peptone from fibrin; 0.200 gram  $NaCl$ .

<sup>4</sup> This *Journal*, xi, p. 507, 1912.

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The solution was slightly alkaline to alizarine, acid to litmus. Two-cubic-centimeter portions were taken for amino nitrogen determinations. The results of the determinations are given in the following table.

TABLE II.

PERIOD OF ACTION	N <sub>2</sub> GAS	TEMPERATURE	PRESSURE
<i>hours</i>	<i>cc.</i>	<i>deg. C.</i>	<i>mm.</i>
0	0.66	22	768
3	0.69	22	768
24	0.70	20	766
72	0.82	26	766

Within the period required to decompose urea (sixteen hours) the action of the extract on the peptone is barely discernible.

To test the effect of the enzyme on amino-acids, the artificially digested meat termed "ereptone" and manufactured at Hoechst a. M. according to Abderhalden's method was used as a substrate. The decomposition of the original proteins into amino-acids was almost complete in the preparation used, as heating for twenty-four hours at 100° with 20 per cent hydrochloric acid increased the amino nitrogen from 63 per cent only up to 67 per cent of the total nitrogen. The use of such a preparation, which probably contains all of the amino-acids found in the body, and in about the proportions in which they exist in the body as a whole, affords a more practical test for the purpose of the experiment than would the utilization of some of the individual amino-acids. The solution contained: 25 cc. H<sub>2</sub>O; 10 cc. soy bean extract; 0.200 gram ereptone (containing 12.8 per cent N); 0.200 gram NaCl; 0.5 cc. 0.1 N NaOH to render the solution just alkaline to alizarine. Amino determination on 2-cc. portions gave:

At once.....1.60 cc. N<sub>2</sub> at 19°, 767 mm.  
After sixteen hours.....1.59 cc. N<sub>2</sub> at 19°, 771 mm.

No deamination whatever occurred.

To test the extract for its ability to hydrolyze hippuric acid the following solution was prepared: Kahlbaum's hippuric acid, 0.200 gram; 0.1 N NaOH (1 equivalent), 11.1 cc; H<sub>2</sub>O, 10 cc; soy bean extract, 10 cc.

The solution was acid to litmus, alkaline to Congo and alizarine. Two-cubic-centimeter portions were taken for analysis. The results were:

At once.....	0.20 cc. N <sub>2</sub> at 21°, 764 mm.
After sixteen hours.....	0.24 cc. N <sub>2</sub> at 21°, 764 mm.

The increase of 0.04 cc. indicates the hydrolysis of 2 per cent of the hippuric acid present. Whether this was due to the action of the extract or to spontaneous splitting of the hippuric acid was not determined. The effect is, in any case, negligible so far as its influence on urine analyses is concerned.

All the above experiments were repeated, with similar results. They show that the soy bean extract, under the conditions used for complete decomposition of urea, does not hydrolyze casein, nor, to a significant extent, peptone or hippuric acid, nor does it deaminate amino-acids.

*Method for free amino-acid nitrogen.*

The proportion of extract necessary to completely hydrolyze urines of the maximum concentration is determined as described on p. 128. To 25 cc. of urine in a 50-cc. flask one adds the required amount of extract (usually about 15 cc. with the beans which we used) and lets the mixture stand for about one and a half times the interval which has been found sufficient to effect the maximum decomposition of urea, as observed by titration of the ammonia. These conditions assure decomposition of the last traces of urea. At the end of the digestion period 10 cc. of a 10 per cent suspension of calcium hydrate are added, and the mixture is shaken and diluted up to the 50 cc. mark. It is then filtered through a dry folded filter, and 20 cc. of the alkaline filtrate are concentrated in a Jena glass dish to dryness on the water bath, this process driving off all the ammonia (hippuric acid is not appreciably affected by this treatment). The residue is moistened with 1 cc. of 50 per cent acetic acid, washed into a 10-cc. measuring flask, and diluted to the mark. One uses the entire solution for a determination in the larger amino apparatus, or 2 cc. for duplicates in the smaller. The reaction period with the nitrous acid should be kept as short as possible for the reasons given on p. 126, and the

## 132 Determination of Amino Nitrogen in Urine

correction for the amines other than amino-acids can be made in the same manner.

The results tabulated below were obtained with the same urines used for determination of total amino nitrogen. The amount of enzyme used was 15 cc.; the time allowed for it to act, five hours at 36°, three hours having been the period in which the proportion of extract used gave a maximum yield of ammonia in a previous test.

TABLE III.

*Free amino nitrogen. All determinations at 26°, 760 mm. Reaction period two and a half minutes.*

NO.	TOTAL N PER 100 CC. URINE	N GAS	CORRECTED FOR AMINES OTHER THAN AMINO-ACIDS	AMINO N PER 100 CC. URINE		PER CENT OF TOTAL N IN FORM OF AMINO-ACID NH <sub>2</sub>	
				Uncor- rected	Corrected (Amino-acid N only)	Uncor- rected	Corrected
	grams	cc.		mgms.	mgms.		
1	1.211	0.36	0.28	9.7	7.5	0.80	0.62
		0.34	0.26	9.2	7.2	0.76	0.58
2	1.750	0.48	0.35	13.0	9.8	0.74	0.56
		0.48	0.35	13.0	9.8	0.74	0.56
3	0.833	0.31	0.25	7.7	6.8	0.93	0.82
		0.31	0.25	7.7	6.8	0.93	0.82
4	1.747	0.55	0.33	15.0	8.9	0.85	0.51
		0.54	0.33	15.0	8.9	0.83	0.51
5	1.309	0.28	0.18	8.0	5.0	0.61	0.38
		0.28	0.18	8.0	5.0	0.61	0.38

The control solution, containing 15 cc. of extract with 25 cc. of water in place of the urine, gave 0.20 cc. of nitrogen gas during the first two and a half minutes of the reaction at 26°, and 0.05 cc. during the second two and a half minutes. These amounts, which were found without appreciable deviation in several duplicates, were subtracted from the volumes of gas read at each determination. The corrections for the volume of gas evolved by amines other than amino-acids were made as described on p. 126. Duplicate amino determinations with 2-cc. portions were made in each case with the micro-apparatus.

It will be noted that the amount of nitrogen evolved by amines other than amino-acids is practically the same, amounting to 0.2-0.3 per cent of the total nitrogen of the urine, whether the urea was removed by hydrolysis with sulphuric acid in the autoclave, or by the urease. When this correction is determined, we believe that the methods for both free and total amino nitrogen described above give with a close degree of approximation the actual amount of amino nitrogen present in the form of amino-acids. As the correction is relatively small and constant, it is probable that in most work, where comparative results chiefly are desired, it will be unnecessary to take it into account.

#### *Hippuric acid.*

Henriques and Sørensen have ascertained the conditions for the complete extraction of hippuric acid from urine with ethyl acetate, its subsequent hydrolysis to glycocoll and benzoic acid with hydrochloric acid, and its determination by titration of the amino nitrogen of the glycocoll by the formol method.<sup>5</sup> The same methods can be applied, making the final determination by the gasometric method instead of the formol titration. When the gasometric method is applied, the results must be multiplied by the factor 0.93, as glycocoll, unlike the other amino-acids, gives off several per cent more gas than the volume corresponding to its nitrogen content. Using this factor, however, one can obtain without trouble results accurate to within 1 per cent of the total glycocoll determined. The gasometric method has some advantages over the formol method,<sup>6</sup> and in this case should be particularly convenient because it simplifies the process of extraction by permitting one to work with small volumes of urine. As work from Henriques and Sørensen hardly requires confirmation of its reliability, and the substitution of the gasometric for the formol method in the final determination is an obvious modification, a more detailed discussion here appears unnecessary.

<sup>5</sup> *Zeitschr. f. physiol. Chem.*, lxiii, p. 27, 1910; lxiv, p. 120, 1911.

<sup>6</sup> *This Journal*, xii, p. 302, 1912.

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### CONCLUSION.

The previously published process of determining the total amino-acid nitrogen (free amino-acids+conjugated amino-acids in the form of hippuric acid, peptides, proteins, etc.) has been simplified so that the operation is much shortened and the more laborious parts, boiling off ammonia and washing bulky precipitates, are dispensed with. The free amino-acids alone can readily be determined after decomposition of the urea with soy bean urease, which hydrolyzes urea completely without either freeing conjugated amino-acids or deaminizing free ones. The applicability of the gasometric method for the determination of hippuric acid is indicated.



## RESEARCHES ON PURINES. XIII.<sup>1</sup>

### ON 2,8-DIOXY-1,6-DIMETHYLPURINE AND 2,6-DIOXY-3,4-DIMETHYL-5-NITROPYRIMIDINE ( $\alpha$ -DIMETHYLNITROURACIL).<sup>2</sup>

BY CARL O. JOHNS AND EMIL J. BAUMANN.

(From the Sheffield Laboratory of Yale University.)

(Received for publication, August 26, 1913.)

We find that an aqueous solution of the sodium salt of 2-oxy-4-methyl-5-nitro-6-aminopyrimidine<sup>3</sup> (I) reacts readily with dimethylsulphate and gives an 80 per cent yield of the corresponding dimethyl derivative. It seemed probable that the compound thus formed was 2-oxy-3,4-dimethyl-5-nitro-6-aminopyrimidine (II), because, in our previous work on alkylations of pyrimidines which contained oxygen in position 2 and an amino or alkylamino group in position 6 we found that the alkyl group entered position 3 in the pyrimidine ring.<sup>4</sup> The following experiments show conclusively that in the case now under consideration the alkyl group also entered position 3.

The substance obtained by methylating 2-oxy-4-methyl-5-nitro-6-aminopyrimidine was heated with 25 per cent sulphuric acid under pressure. This treatment removed the amino group and a good yield of a 2,6-dioxy-5-nitro-dimethyl-pyrimidine was obtained. Two such compounds can exist in which one of the methyl groups is attached to nitrogen in the urea grouping of the pyrimidine ring and the other methyl group attached to the carbon atom in position 4, namely, 2,6-dioxy-1,4-dimethyl-5-nitropyrimidine (VII) and 2,6-dioxy-3,4-dimethyl-5-nitropyrimidine (III). Lehman<sup>5</sup> ob-

<sup>1</sup> Johns and Baumann: this *Journal*, xv, p. 515, 1913. The present investigation was aided by a grant from the Bache fund.

<sup>2</sup> Behrend and Dietrich: *Ann. d. Chem. (Liebig)*, cccix, p. 266, 1899; Behrend and Thurm: *ibid.*, ccxxiii, p. 163, 1902.

<sup>3</sup> Johns: *Amer. Chem. Journ.*, xli, p. 60, 1909.

<sup>4</sup> Johns: this *Journal*, xi, p. 75, 1912; xiv, p. 3, 1913.

<sup>5</sup> Lehman: *Ann. d. Chem. (Liebig)*, ccliii, p. 84, 1899.

tained one of the above compounds by the action of methyl iodide on the potassium salt of nitromethyluracil.<sup>6</sup> His compound melted at 149°C. and the structure assigned to it was 2,6-dioxy-1,4-dimethyl-5-nitropyrimidine<sup>7</sup> (VII). Our 2,6-dioxy-5-nitro-dimethylpyrimidine melts at 191°C. Hence, it cannot be identical with the compound obtained by Lehman and if the correct structure has been assigned to his compound ours must be 2,6-dioxy-3,4-dimethyl-5-nitropyrimidine (III).

Behrend and Köhler<sup>8</sup> have shown that fuming nitric acid not only nitrates 4-methyluracil (X) but also oxidizes the methyl group forming 2,6-dioxy-4-carboxyl-5-nitropyrimidine (XI) and that this latter compound loses carbon dioxide with the consequent formation of 2,6-dioxy-5-nitropyrimidine or nitrouracil (XII).

We found that 2,6-dioxy-5-nitro-dimethylpyrimidine was also oxidized by fuming nitric acid and that the 2,6-dioxy-3-methyl-4-carboxyl-5-nitropyrimidine (VI) lost carbon dioxide which resulted in the formation of 2,6-dioxy-3-methyl-5-nitropyrimidine (IX). The structure of this compound has been firmly established by the work of Behrend and his collaborators.<sup>9</sup> It melts at 255°C. and contains one molecule of water of crystallization and is therefore readily distinguished from its isomer 2,6-dioxy-1-methyl-5-nitropyrimidine<sup>10</sup> (VIII) which melts at 263°C. and does not contain water of crystallization. The compound obtained by us contained water of crystallization and melted at 255°C. It was therefore 2,6-dioxy-3-methyl-5-nitropyrimidine. Hence in methylating 2-oxy-4-methyl-5-nitro-6-aminopyrimidine the methyl group entered position 3 and the compound formed was 2-oxy-3,4-dimethyl-5-nitro-6-aminopyrimidine (II).

The latter compound was reduced rapidly by the action of freshly precipitated ferrous hydroxide but the reaction was not smooth. After isolating about 40 per cent of the calculated weight of 2-oxy-3,4-dimethyl-5,6-diaminopyrimidine (V) a tarry by-product remained.

<sup>6</sup> Behrend: *Ann. d. Chem.* (Liebig), ccxl, p. 3, 1887.

<sup>7</sup> *Beilstein's Handbuch*, i, p. 1350 (third edition).

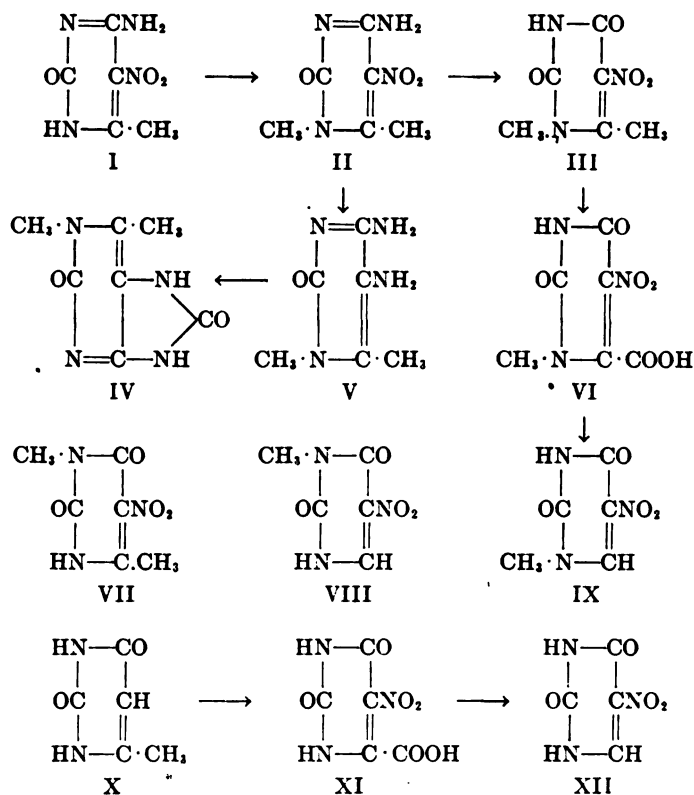
<sup>8</sup> Behrend: *Ann. d. Chem.* (Liebig), cccxix, p. 32, 1885; Köhler: *ibid.*, cccxxv, p. 50, 1886.

<sup>9</sup> Behrend and Thurm: *Ann. d. Chem.* (Liebig), cccxxiii, p. 163, 1902.

<sup>10</sup> *Ibid.*

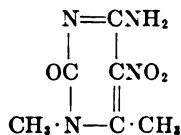
When 2-oxy-3,4-dimethyl-5,6-diaminopyrimidine was heated with urea we obtained an excellent yield of 2,8-dioxy-1,6-dimethylpurine (IV).

These researches will be continued.



#### EXPERIMENTAL PART.

##### *2-Oxy-3,4-dimethyl-5-nitro-6-aminopyrimidine.*



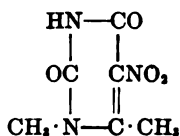
Ten grams of pulverized 2-oxy-4-methyl-5-nitro-6-aminopyrimidine<sup>11</sup> were dissolved in 100 cc. of hot water containing 2.8 grams of sodium hydroxide. After cooling this solution to room temperature, 10 grams of dimethylsulphate were added and the mixture was shaken to keep the dimethylsulphate in suspension. In less than five minutes crystals began to form. The mixture was shaken two or three minutes longer and then allowed to stand until it gave an acid reaction, which usually required less than fifteen minutes. Heat was evolved during the reaction and, after cooling, the precipitate was filtered off and washed with a little cold water and alcohol. The yield was 8.7 grams or 80 per cent of theory. This substance contained but a trace of the original 2-oxy-4-methyl-5-nitro-6-aminopyrimidine and was pure enough for subsequent experiments. The 2-oxy-3,4-dimethyl-5-nitro-6-aminopyrimidine dissolved readily on continued boiling in water and on cooling the solution slowly it crystallized in lustrous prisms that formed radiating clusters or sheaves. These crystals did not have a definite melting point but began to darken at about 170°C. and effervesced at 190° to 195°C. They were moderately soluble in hot alcohol, slightly soluble in boiling benzene and insoluble in ether. They dissolved readily in dilute hydrochloric acid and glacial acetic acid. They formed yellow solutions in strong alkalies and were moderately soluble in ammonium hydroxide. The crystals which were obtained from aqueous solutions possessed a pearly luster. This was lost on drying over sulphuric acid in a desiccator for one or two days though analyses showed that one-half molecule of water of crystallization still remained.

I. 2.0855 grams of substance dried over sulphuric acid for twenty-four hours lost 0.0993 gram at 120°-130°C.

II. 2.8975 grams of substance dried over sulphuric acid for 48 hours lost 0.1375 gram at 120°-130°C.

	Calculated for $C_6H_8O_2N_4 \cdot \frac{1}{2}H_2O$ :	Found:	
		I	II
H <sub>2</sub> O.....	4.66	4.76	4.73
	Calculated for $C_6H_8O_2N_4$ :	Found:	
		I	II
N.....	30.43	30.38	30.54

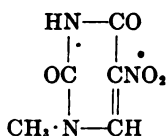
<sup>11</sup> Johns: *loc. cit.*

*2,6-Dioxy-3,4-dimethyl-5-nitropyrimidine.*

Three grams of 2-oxy-3,4-dimethyl-5-nitro-6-aminopyrimidine from the above experiment were dissolved in 20 cc. of 25 per cent sulphuric acid and the solution was heated in a sealed tube at 160°C. for two hours. When the contents of the tube were cooled a deposit of long, slender prisms was obtained. A second crop was isolated by neutralizing the filtrate with barium hydroxide, filtering, and concentrating this filtrate. The yield was 75 per cent of theory. The crude substance melted at 186° to 190°C. When recrystallized from alcohol the melting point was 191°C. It was easily soluble in hot water and on cooling the solution it crystallized rapidly in slender prisms. It also dissolved readily in hot alcohol, slightly in benzene but did not dissolve in ether. Dilute alkalis dissolved it easily.

	Calculated for $\text{C}_6\text{H}_7\text{O}_4\text{N}_3$ :	Found:	
		I	II
N.....	22.70	22.61	22.62

*The oxidation of 2,6-dioxy-3,4-dimethyl-5-nitropyrimidine with nitric acid. The formation of 2,6-dioxy-3-methyl-5-nitropyrimidine.*<sup>12</sup>



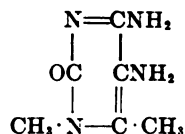
One gram of 2,6-dioxy-3,4-dimethyl-5-nitropyrimidine was dissolved in 10 cc. of nitric acid of specific gravity 1.5 and 2 cc. of concentrated sulphuric acid were added. The solution was heated on the water bath for one and one-half hours. Oxidation took place with effervescence and the liberation of brown fumes. The solution was diluted with water and the acids were neutralized with ammonia. On evaporating to dryness and washing with cold

<sup>12</sup> Behrend and Thurm: *Ann. d. Chem.* (Liebig), cccxxiii, p. 164, 1902.

water to remove salts, there remained a crystalline substance that weighed 0.2 gram. This melted at 254° to 255°C. and when recrystallized from water it melted sharply at 255° to 256°C. The crystals contained water of crystallization and when mixed with a pure sample of 2,6-dioxy-3-methyl-5-nitropyrimidine the melting point remained the same. Hence, the methyl group attached to nitrogen was in position 3 in the pyrimidine ring. The substance was dried at 120° to 130°C.

	Calculated for	Found:
	C <sub>5</sub> H <sub>5</sub> O <sub>4</sub> N <sub>3</sub> :	
N.....	24.56	24.44

*2-Oxy-3,4-dimethyl-5,6-diaminopyrimidine.*

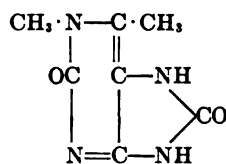


Five grams of 2-oxy-3,4-dimethyl-5-nitro-6-aminopyrimidine were dissolved in a mixture of 50 cc. of concentrated ammonia and 75 cc. of water by warming gently. This solution was cooled to room temperature and a hot, concentrated aqueous solution of 53 grams of crystallized ferrous sulphate was added gradually. Reduction took place rapidly and was accompanied with the liberation of heat. A solution of 63 grams of barium hydroxide was added to precipitate the sulphate and the excess of baryta was removed by adding ammonium carbonate. The mixture was well shaken and filtered after standing for an hour. The filtrate was evaporated to a small volume on the water bath and then cooled. A dark crystalline mass separated. This was dissolved in hot water and decolorized with blood coal. The diaminopyrimidine was thus obtained pure and colorless in the form of burrs that consisted of small plates. The yield was about 40 per cent of theory. The crystals possessed a pearly luster. They were very soluble in hot water and crystallized well on cooling the solution. They were moderately soluble in boiling alcohol but did not dissolve in benzene or ether. Dilute acids dissolved them easily. They did not exhibit a definite melting point but began to decompose at about 230°C. When an aqueous

solution of this substance was added to a cold ammoniacal silver solution a white gelatinous precipitate resulted and, on heating, a silver mirror was produced.

	Calculated for $C_8H_{10}ON_4$	Found:
N.....	36.36	36.37

*2,8-Dioxy-1,6-dimethylpurine.*



Two grams of 2-oxy-3,4-dimethyl-5,6-diaminopyrimidine and 2 grams of urea were pulverized together and the mixture was heated for an hour at 170° to 180°C. in an oil bath. The mass melted to a liquid and frothing occurred while there was a copious evolution of ammonia. After some twenty-five minutes the reaction subsided and the mixture became a solid mass. After cooling, the reaction-product was dissolved in dilute ammonia and the solution was clarified with blood coal. After boiling off most of the ammonia, the solution was acidified with acetic acid whereupon crystals of the purine began to separate as the solution cooled. After two hours the crystals were filtered off. Another crop was obtained by concentrating the filtrate. The yield was 2 grams or 85 per cent of the calculated weight. The portion used for analysis was recrystallized from water. This purine dissolves in about 60 parts of boiling water and is slightly soluble in hot alcohol but does not dissolve in benzene or ether. It dissolves readily in hydrochloric acid or alkalies. It crystallizes from water in burrs composed of small prisms that contain one molecule of water of crystallization. It decomposed at 260° to 265°C. It did not form a difficultly soluble picrate or barium salt. Its water solution gave a gelatinous precipitate with mercuric chloride. This was soluble in hot water but reappeared on cooling the solution. With an ammoniacal silver solution a white precipitate was obtained. This did not darken when the contents of the test tube were boiled. Nitric acid oxi-

dized the purine readily and on careful evaporation a yellow crust remained. This became rose colored when treated with alkalies.

0.8798 gram of substance lost 0.0825 gram at 120° to 130°C.

	Calculated for $C_7H_5O_2N_4.H_2O$ :	Found:
$H_2O$ .....	9.09	9.29
	Calculated for $C_7H_5O_2N_4$ :	Found:
N.....	31.11	31.32



## POLYATOMIC ALCOHOLS AS SOURCES OF CARBON FOR LOWER FUNGI.

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(From the Chemical Section of the Iowa Agricultural Experiment Station.)

(Received for publication, August 30, 1913.)

The carbon nutrition of the lower fungi has been studied quite extensively. The diversity of simple organic substances which molds are able to utilize as sources of carbon is indeed surprising. Not only do the naturally-occurring sugars supply the carbon requirements of these fungi, but many other substances, among them laboratory products not known to occur in nature, appear to be more or less readily utilized by these organisms. The vigor of the culture, however, may vary considerably with the nature of the substrate. Many substances on which only a scant growth can be obtained have been reported in the literature as available sources of carbon. It is of course impossible to express availability of a particular substrate for a given organism on an adequate quantitative basis, yet some distinction should be made between a sparse growth and a vigorous culture.

The cultures herein described were made for the purpose of determining differences in availability in the series of polyatomic alcohols. The substances selected represent a series differing progressively by the group CHOH. They all occur in nature, the first three in the form of esters, the others in the free state. Isomeric synthetic products will not be considered here. The series represents therefore substances containing one to six alcohol radicals and a similar number of carbon atoms, and may be designated by the general formula  $C_nH_{2n+2}O_n$ . Following is the list:<sup>1</sup>

NUMBER OF CARBON ATOMS AND ALCOHOL RADICALS	SUBSTANCE	NUMBER OF CARBON ATOMS AND ALCOHOL RADICALS	SUBSTANCE
1	Methyl alcohol	5	Adonitol
2	Ethylene glycol	6	Mannitol
3	Glycerol	6	Dulcitol
4	Erythritol	6	Sorbitol

<sup>1</sup>Several of these substances have been reported by Emmerling (*Centralbl. f. Bakt.*, x, II, p. 273) as available for *Aspergillus niger*, but quantitative differences were not considered.

These substances were introduced into Czapek's medium in place of the usual sugar. No other source of carbon was present. Inoculation was made with the spores of pure cultures, and the tubes were allowed to remain in the dark at room temperature. The cultures were examined at the end of the first, second and third week. The following notation will be used to designate the appearance of the culture:

+++.....good normal culture  
 ++.....medium growth  
 +.....slight growth  
 G.....germination or submerged hyphae only  
 O.....no growth

ORGANISM	AGE OF CULTURE IN WEEKS	SOURCE OF CARBON									
		None	Methyl alcohol	Ethylene glycol	Glycerol	Erythritol	Adonitol	Sorbitol	Dulcitol	Mannitol	Sucrose
<i>Aspergillus niger</i>	1	O	O	O	+++++		G	+++	+++	+++	+++
	2	O	O	G	+++++		G	+++	+++	+++	+++
	3	O	O	G	+++++		G	+++	+++	+++	+++
<i>Aspergillus clavatus</i>	1	O	O	G	+++	+	+	+	G	+++	+++
	2	O	O	G	+++	+	+	+	G	+++	+++
	3	O	O	G	+++	+	+	++	G	+++	+++
<i>Aspergillus fumigatus</i>	1	O	O	G	+++++		+	+++	+++	++	+++
	2	O	O	+	+++++	++		+++	+++	+++	+++
	3	O	O	+	+++++	++		+++	+++	+++	+++
<i>Penicillium expansum</i>	1	O	O	G	+++++		+	+++	+++	++	+++
	2	O	O	+	+++++	++		+++	+++	+++	+++
	3	O	O	+	+++++	++		+++	+++	+++	+++
<i>Fusarium oxysporium</i>	1	O	O	+	+++	G	+++	+++	+++	+++	+++
	2	O	O	++	+++	G	+++	+++	+++	+++	+++
	3	O	O	++	+++	G	+++	+++	+++	+++	+++
<i>Cladisporium herbarum</i>	1	O	O	G	+++	+	+	+	G	+++	+++
	2	O	O	G	+++++	++		+	G	+++	+++
	3	O	O	G	+++++	++		+	G	+++	+++
<i>Penicillium roqueforti</i>	1	O	O	G	+++	+	++	++	+	++	+++
	2	O	O	+	+++++	++		+++	++	+++	+++
	3	O	O	+	+++++	++		+++	++	+++	+++
<i>Penicillium camemberti</i>	1	O	O	G	++	G	+	+++	++	+	++
	2	O	O	G	+++++		+	+++	+++	+++	+++
	3	O	O	+	+++++		+	+++	+++	+++	+++

It will be noted that the first two members of the series are not capable of producing normal cultures. Glycerol is readily available and gives cultures equal in vigor to those grown on cane sugar. With increasing carbon, however, the availability does not increase, as might perhaps be expected. Adonitol, for example, does not compare favorably with glycerol or even erythritol, and two of the hexatomic alcohols fail to yield cultures equal to those on glycerol.

It will be noted that the alcohols beginning with erythritol contain asymmetric carbon atoms. But considering the fact that glycerol is not asymmetric, no connection can be established between availability and carbon asymmetry. On the other hand, there may be some relation between availability and the nature of the intermediate oxidation products, since all the substances which are available, including glycerol, yield oxidation products containing one or more asymmetric carbon atoms.

The writer takes pleasure in acknowledging his indebtedness to Dr. A. W. Dox, at whose suggestion this work was undertaken.



## THE COMPARATIVE COMPOSITION OF HUMAN MILK AND OF COW'S MILK.

BY EDWARD B. MEIGS AND HOWARD L. MARSH.

(From the Robert Hare Chemical Laboratory of the University of Pennsylvania and the Wistar Institute of Anatomy and Biology.)

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### INTRODUCTION.

The following article is an account of work done by Arthur V. Meigs, Howard L. Marsh, William H. Welker and W. L. Croll on the chemical analysis of human milk and of cow's milk. The work was carried out in the Robert Hare Chemical Laboratory of the University of Pennsylvania and was to a large extent supervised by John Marshall, the Director of that laboratory. The present account has been written by Edward B. Meigs, in collaboration with Howard L. Marsh, after the death of Arthur V. Meigs, on January 1, 1912.

The subject of milk analysis was taken up by Arthur V. Meigs more than thirty years ago with the idea of discovering how cow's milk should be modified in order to make a proper food for very young infants. On the basis of analyses made in 1881-1884, Meigs devised a food which he afterward used in his practice with great success. He published an account of his early investigations in book form in 1885,<sup>1</sup> and has since published a number of smaller articles on the subject.<sup>2</sup> In 1908 he again began chemical work on the subject which he continued until his death in 1912. A short preliminary account of some of the results of this later work appeared in 1911;<sup>3</sup> and since that time

<sup>1</sup> Arthur V. Meigs: *Milk Analysis and Infant Feeding*, Philadelphia, 1885.

<sup>2</sup> Arthur V. Meigs: *Transactions of the College of Physicians of Philadelphia*, Third Series, viii, p. 139, 1885; *Ibid.*, xxiv, p. 136, 1902; *Archives of Pediatrics*, December, 1889; *Feeding in Early Infancy*, Philadelphia, 1896.

<sup>3</sup> Arthur V. Meigs and Howard L. Marsh: *The Medical Record*, December 30, 1911.

the authors of this article have been endeavoring to coördinate the other results and to prepare them for publication.

Meigs' work on milk in 1881 consisted in an attempt to compare human milk and cow's milk in their content of protein (or "casein"),<sup>4</sup> fat, lactose, water and salts. The chief outcome of the work was the conclusion that human milk contains less than half as much protein as cow's milk and about 50 per cent more lactose. In other respects Meigs' results were not in sharp disagreement with those of his predecessors, but his figures for protein and lactose were quite different from those which were usu-

TABLE I.

*The average composition of human milk and cow's milk according to Meigs' early analyses, and those of certain well-known authors whose results were available in 1881. The various constituents are given as percentages of the whole milk.*

AUTHOR	WATER		PROTEIN		FAT		LACTOSE		ASH	
	Human milk	Cow's milk	Human milk	Cow's milk	Human milk	Cow's milk	Human milk	Cow's milk	Human milk	Cow's milk
Lehmann* . .			3.5	4.5			4 to 6			
Gorup-										
Besanez† .	88.908	84.28	3.924	4.35	2.666	6.47	4.364	4.34	0.138	0.63
Meigs‡ . . . . .	87.163	87.780	1.046	3.022	4.283	3.759	7.407	4.949	0.101	0.488

\* Lehmann: *Physiological Chemistry*, Cavendish Society translation, London, 1851, vol. I. p. 383, vol. II, p. 341.

† Gorup-Besanez: *Lehrbuch der physiologischen Chemie*, Braunschweig, 1878, pp. 421 and 424: the figures for human milk are those quoted from Vernois and Becquerel, and are averages from 89 analyses.

‡ Meigs: *Milk Analysis and Infant Feeding*, Philadelphia, 1885, pp. 34 and 36.

ally given at that time. A comparison of Meigs' figures with some of the best known figures which were available in 1881 is given in Table I.

Meigs was of course familiar with later analyses which had not at that time been quoted in text-books. In these analyses the protein of human milk was variously given at from 0.215 to

<sup>4</sup> The word "casein" was, at that time, very generally used to designate all the proteins of milk; it now means, as is well known, a particular protein body which constitutes about 80 per cent of the total protein of cow's milk and a somewhat less proportion of the protein of human milk. In the subsequent discussion the words "casein" and "protein" will be used in their modern senses.

over 7 per cent, while the figures for lactose ranged from 1.921 to 8.805 per cent.

In the milk analyses which have been published since 1881, the figures given to represent the percentage of protein and lactose in cow's milk have not varied widely from those published by Gorup-Besanez. But the percentages of these constituents in human milk have been quite variously given, as Table II shows; in the more modern analyses the results are quite near to those reached by Meigs in 1881.

It is generally agreed among physiological chemists at present that the fat, lactose, ash and total nitrogen of milk can be quantitatively determined satisfactorily. The difficulties of milk anal-

TABLE II.

*The average composition of human milk according to analyses published since 1881. The constituents are given as percentages of the whole milk.*

	WATER	PROTEIN	FAT	LACTOSE	ASH
Munk and Uffelmann*....	89.2	2.1	3.4	5.0	0.2
König†.....	87.4	2.3	3.8	6.2	0.3
Heubner‡.....		1.03	4.07	7.03	0.21
Camerer and Söldner§....		1.27	3.91	6.52	0.22

\* Munk and Uffelmann: *Ernährung des gesunden und kranken Menschen*: Wien and Liepsig, 1887, p. 269.

† Quoted by Camerer and Söldner: *Zeitschr. f. Biol.*, xxxiii, 1896, p. 43.

‡ Heubner: *Berl. klin. Wochenschr.*, 1894, Nos. 37 and 38.

§ Camerer and Söldner: *Zeitschr. f. Biol.*, xxxvi, Table II, pp. 280 and 281, 1898. The figures are average figures for milk between the 20th and 40th day post partum. The figure for protein is obtained by multiplying the figure for total nitrogen given by the authors by the factor 6.25.

ysis lie in the determination of the protein and of the unknown constituents. Meigs and his collaborators have, in the first place, determined the quantities of fat, lactose, ash and total nitrogen in a number of samples of human milk and cow's milk; they then attacked the question of the protein and unknown constituents. It will be convenient to divide this article into two parts, in the first of which will be given the results on the easily determinable constituents of milk; and in the second, the work on the protein and unknown substances; this latter work was still in a very fragmentary state at the time of Meigs' death. It will be well to begin with a description of the samples of milk used in the various studies.

Each of the samples of human milk numbered 1, 2, 4, 6, 7, 8 and 10 was made up of milk from several women; each of the other samples was composed of the milk from one woman. The composite samples of human milk came from women in maternity hospitals, and all were taken between the fourth and ninth days after delivery. The other samples of human milk may be described as follows:

No. 3. Colostrum—collected up to and including the fourth day after delivery; maternity hospital patient.

No. 5. Milk collected near the end of lactation, eighteen months or more post partum; woman in moderate circumstances. The flow of milk had almost ceased so that only very little could be obtained.

No. 9. Milk collected during the fifth month after delivery; woman in good circumstances.

The cow's milk came partly from thoroughbred Guernsey cows and partly from grade cows; samples 1, 9, 13 and 20 are the former, the others, the latter. Each of the samples of cows' milk except Nos. 1, 9, 13 and 20 was made up of milk from several cows, and the periods of lactation have not been accurately determined. All the samples may, however, be taken to represent milk from the middle period of lactation, from two to six months after delivery.

In preparing the work for publication, the data obtained from a few samples of milk have been left entirely out of consideration, either because they contained obvious analytical errors, or else because the samples were used for answering subsidiary questions which had no bearing on the composition of milk.

#### PART I. STUDIES ON THE AMOUNTS OF ASH, LACTOSE, FAT AND NITROGEN IN HUMAN MILK AND IN COW'S MILK.

##### *Methods of experimentation.*

The ash determinations were made by igniting the dry residues from the samples cautiously at low temperatures in platinum dishes.

The nitrogen determinations were made by the Kjeldahl method.

The lactose was determined by Fehling's method as follows: 25 cc. of cow's milk or 15 cc. of human milk were diluted to 400 cc. in a 500 cc. graduated flask. Fifteen cc. of  $\frac{N}{4}$  NaOH and 10 cc. of  $\text{CuSO}_4$  of the strength used in a Fehling's solution were added and the volume of the liquid in the flask was diluted to 500 cc. with water.

After this mixture had been well shaken, it was filtered through a dry filter into a dry flask. One hundred cc. of this solution were added to 50 cc. of hot Fehling's solution and the mixture was boiled for six minutes. It was then quickly filtered through an alundum crucible and washed with about 600 cc. of boiling water. The resulting cuprous oxide in the crucible was dissolved with nitric acid and the solution poured into the beaker which had been used in the reduction. The crucible was then thoroughly washed by passing hot water through it and this wash water was added to



the nitric acid solution which had been poured from the crucible. This nitric acid solution of the cuprous oxide was evaporated on a water bath until free from nitric acid. It was then dissolved with acetic acid and water, 8 grams of zinc acetate were added and the liquid was transferred to a small glass-stoppered bottle, 4 grams of potassium iodide were added and the solution titrated against  $\frac{N}{10}$  sodium thiosulphate with starch as an indicator.

The amount of lactose corresponding to the amount of copper found was ascertained by referring to a table given in Bulletin No. 107 (revised) of the U. S. Department of Agriculture, Bureau of Chemistry, pp. 48 and 49. The figures represent lactose plus one molecule of water of crystallization.

Fat was determined by a modification of the method recommended by Meigs in 1885.<sup>5</sup> To 10 cc. of milk in a 100 cc. glass-stoppered cylinder were added 20 cc. of distilled water and 20 cc. of ethyl ether and the mixture shaken for five minutes. Twenty cc. of 95 per cent ethyl alcohol were then added and the whole again shaken for five minutes. The cylinder was then allowed to stand until its contents separated into two distinct layers. The upper layer was removed by the specially designed pipette shown in figure 1, 5 cc. of ethyl ether were added in such a way as to wash down the sides of the cylinder, this was removed and added to the upper layer previously removed, and the washing of the sides of the cylinder and of the top of the lower layer of the mixture was repeated five times in order to remove all the fat. The upper layer from the mixture plus washings was then evaporated to dryness on a water bath and the residue desiccated to constant weight over sulphuric acid at room temperature. The weight of the residue may be taken to be very nearly that of the fat contained in the milk.

The residue in question was in a number of cases treated with dry ether, and it was found that a minute portion of it usually failed to dissolve. This insoluble portion was, however, never as much as 3 per cent of the weight of the original residue; it amounted on the average to about 1 per cent. It was shown by Fehling's and by the phenyl-hydrazine tests to consist largely of lactose.

The fat as obtained by Meigs' method from two portions of a sample of cow's milk (No. 14) was analyzed for nitrogen: 0.0094 per cent and 0.0064 per cent respectively of nitrogen was found. It is probable that a considerable portion of this comes from the ether-soluble lipoids.

In one sample of human milk (No. 8) the ash, as determined in the fat obtained by Meigs' method, was found to be 0.0081 per cent of the weight of the whole milk.

In other experiments the fat as obtained by Meigs' method in certain samples of milk was compared with the fat as obtained by the Soxhlet extraction of the dried solids. These results have already been published:<sup>6</sup> in twelve determinations on human milk the fat as determined by Meigs'

<sup>5</sup> Meigs: *Milk Analysis and Infant Feeding*, Philadelphia, 1885.

<sup>6</sup> Hawk: *Practical Physiological Chemistry*, Philadelphia, 1912, p. 437.

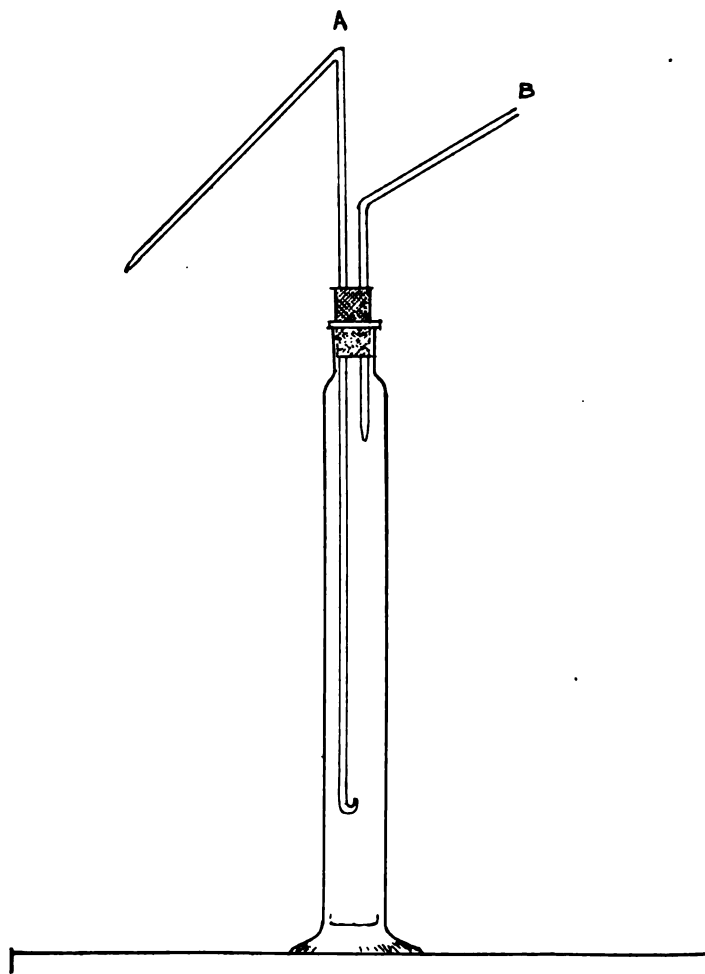


FIG. 1. PIPETTE ARRANGED TO REMOVE THE UPPER LAYER IN MEIGS' METHOD OF FAT EXTRACTION.

The end of the tube *A* is placed at the surface of the division between the two layers of the mixture of milk treated with the water, alcohol and ether mixture and the upper layer is then forced out through it by forcing air into the space above the mixture through tube *B*.

method averaged 0.017 per cent less than by the Soxhlet method; and in seven determinations on cow's milk, 0.019 per cent less. It is by no means certain, however, that the figures for fat obtained by the Soxhlet method in these experiments are more nearly correct than those obtained by Meigs' method; for the prolonged treatment of the milk residue with ether in the Soxhlet apparatus is apt to dissolve substances other than fat, and thus give a fictitious additional weight to the fat extract.<sup>7</sup>

The result of this investigation has been that the product obtained by Meigs' method of extracting the fat from milk contains 98 per cent or more of material soluble in dry ether. The results as obtained by Meigs' method are practically the same as those obtained by the Soxhlet extraction; and Meigs' method is much quicker and subjects the milk to less treatment which is apt to produce changes in the protein and carbohydrate constituents of the fluid.

Meigs' method of determining the fat in milk was used as a matter of routine in this investigation, and the figures for fat in the tables represent the dry weight of the material obtained from the "upper layer" and washings in the procedure which has just been described. In some cases, however, the fat was determined by the Soxhlet extraction as well as by Meigs' method. The figures obtained by the Soxhlet extraction are given in a footnote to Table III.

The results of the determinations of the ash, fat, lactose and total nitrogen of human milk and cow's milk are shown in Table III.

These data agree with those of Camerer and Söldner<sup>8</sup> and with most of the more recent work on milk analysis in showing the marked difference between human milk and cow's milk in respect to their content of nitrogen and lactose. Human milk contains roughly 50 per cent more lactose than cow's milk, and decidedly less than half as much nitrogen. The figures in Table III agree also, so far as they go, with the conclusion of Camerer and Söldner<sup>9</sup> that the lactose in human milk increases, while the nitrogen decreases with the progress of lactation.

<sup>7</sup> Croll has published in detail his comparison of the results obtained by Meigs' method of fat extraction and by the Soxhlet method in the *Biochemical Bulletin* for June, 1913.

<sup>8</sup> Camerer and Söldner: *Zeitschr. f. Biol.*, xxxvi, Table II, pp. 280 and 281, 1898.

<sup>9</sup> *Ibid.*

TABLE III.

The quantities of ash, fat, lactose and total nitrogen in human milk and cow's milk given as percentages of the weight of the whole milk. Duplicate analyses were carried out on each sample of milk and the table gives the figures obtained in each analysis and the average.

	ASH			FAT			LACTOSE			TOTAL NITROGEN			
	I	II	Average	I	II	Average	I	II	Average	I	II	Average	
<i>Human milk.</i>													
(Milk No. 4.....	0.186	0.189	0.187	2.198	2.210	2.204	6.783	6.759	6.771	0.2209	0.2274	0.2242	
Milk No. 6.....	0.191	0.171	0.181	2.234	2.217	2.225	6.443	6.443	6.443	0.2760	0.2765	0.2765	
5th to 9th day post partum.....	0.151	0.154	0.152	2.863	2.874	2.868	6.379	6.378	6.379	0.1962	0.1951	0.1956	
Milk No. 8.....	0.202	0.191	0.196	2.037	2.019	2.028	6.504	6.504	6.504	0.2642	0.2602	0.2622	
Milk No. 10.....	0.249	0.256	0.252	3.147	3.134	3.140	6.600	6.549	6.574	0.2860	0.2828	0.2844	
Milk No. 9, 5th month post partum.....	0.185	0.175	0.180	4.081	4.081	4.081	7.067	7.094	7.080	0.1645	0.1574	0.1609	
Milk No. 5, collected near the end of lactation.....	0.165	0.162	0.163	0.856	0.850	0.853	7.294	7.305	7.299	0.1965	0.1956	0.1960	
<i>Cow's milk.</i>													
(No. 7.....	0.686	0.676	0.681	4.152	4.139	4.145	4.599	4.641	4.620	5.2880	5.3210	5.304	
No. 8.....	0.720	0.721	0.720	4.176	4.224	4.200	4.632	4.601	4.616	5.5000	5.5300	5.515	
Samples of mixed milk from grade cows {	No. 11.....	0.719	0.660	0.689	3.947	3.936	3.941	4.745	4.737	4.741	5.6000	5.5830	5.591
No. 15.....	0.692	0.694	0.693	4.383	4.384	4.383	4.823	4.823	4.823	5.0420	5.0830	5.062	
No. 16.....	0.725	0.731	0.728	3.785	3.804	3.794	4.713	4.713	4.713	4.9810	4.9700	4.975	
No. 19.....	0.732	0.735	0.733	3.215	3.220	3.220	4.490	4.474	4.482	5.2890	5.1910	5.230	
Samples of milk from single {	No. 1*.....	0.728	0.654	0.691	5.352	5.409	5.395	4.940	5.070	5.005	5.6920	5.5550	5.623
thoroughbred Guernsey {	No. 9.....	0.729	0.739	0.734	4.695	4.689	4.692	4.805	4.819	4.812	4.9590	no dup.	0.4959
cows {	No. 13.....	0.774	0.768	0.771	4.605	4.615	4.610	4.741	4.755	4.748	5.9480	5.9490	5.948

The figures obtained for fat in this sample of milk by the Bomblies method of extraction were I, 5.319; II, 5.344; average, 5.331.

## PART II. THE NATURE AND AMOUNT OF THE PROTEIN AND UNKNOWN SUBSTANCES IN HUMAN MILK AND IN COW'S MILK.

Meigs hoped in his later work to satisfactorily settle the quantities of protein in human milk and in cow's milk, and perhaps even to determine the nature of some of the more important unknown substances, which are supposed to be present in the two secretions. Both these hopes were still unfulfilled at the time of his death, but the work has thrown some further light on the problem; and the results, so far as they go, will be given here.

*Experiments which bear solely on the protein content of milk.*

The casein and globulin were precipitated from several samples of human milk and cow's milk by magnesium sulphate and the albumin was precipitated from the magnesium sulphate filtrates by acetic acid and heat.<sup>10</sup> The nitrogen content was determined in each precipitate separately, and also, in a separate portion, for the whole milk. The results are given in Table IV.

In other samples of milk the colloids were completely precipitated from the whole milk according to Marshall's<sup>11</sup> aluminium hydroxide method. The nitrogen in the colloid precipitates, in the filtrates, and in separate portions of the whole milk was determined. The results are given in Table V.

No very definite conclusions regarding the usual protein content of human milk can be drawn from this part of the work, because the samples of human milk used were so few and from so early a period of lactation. So far as the results go, however, they confirm the view that human milk contains less than half as much protein as cow's milk. The results are interesting also in showing that a large proportion of the nitrogen in early human milk exists in compounds which are not precipitable either by magnesium sulphate or by acetic acid and heat; and the results with Marshall's reagent indicate that most of this "non-precipitable" nitrogen exists in non-colloidal bodies.

<sup>10</sup> For details of these methods see Bulletin No. 107 (revised) of the U. S. Department of Agriculture, 1910, p. 118.

<sup>11</sup> Marshall and Welker: *Journ. Amer. Chem. Soc.*, xxxv, p. 820, 1913.

## Composition of Milk

TABLE IV.

The total nitrogen, the amounts of nitrogen contained in the casein and globulin precipitates and in the albumin precipitates, and the non-precipitable\* nitrogen in human milk and cow's milk given as percentages of the weight of the whole milk.

	TOTAL NITROGEN			NITROGEN IN CASEIN AND GLOBULIN PRECIPITATED BY MAGNESIUM SULPHATE			NITROGEN IN ALBUMIN PRECIPITATED BY ACETIC ACID AND HEAT			NITROGEN NOT PRECIPITATED EITHER BY MAGNESIUM SULPHATE OR BY ACETIC ACID AND HEAT
	I	II	Average	I	II	Average	I	II	Average	
<i>Human milk.</i>										
Sample No. 3 (colostrum).....	0.2885	0.3038	0.2962	0.1600	0.1545	0.1572	0.0437	0.0591	0.0514	0.0876
Sample No. 2 (early milk).....	0.2181	0.2191	0.2186	0.1408	0.1347	0.1377	0.0228	0.0307	0.0267	0.0542
<i>Cow's milk.</i>										
Sample No. 1 from Guernsey cow.....	0.5692	0.5555	0.5623	0.4970	0.4890	0.4925	0.0469	0.0495	0.0482	0.0216
{ No. 2.....	0.5121	0.5149	0.5135	0.4129	0.4109	0.4119	0.0593	0.0579	0.0586	0.0430
Samples of mixed milk from { No. 3.....	0.5355	0.5029	0.5192	0.4347	0.4454	0.4402	0.0564	0.0499	0.0531	0.0259
{ No. 4.....	0.5129	0.5251	0.5190	0.4189	0.4304	0.4246	0.0527	0.0504	0.0515	0.0429
grade cows { No. 5.....	0.5251	0.5019	0.5135	0.4321	0.4137	0.4229	0.0394	0.0458	0.0426	0.0480

\* The figures for the "non-precipitable" nitrogen are obtained by subtracting the sum of the quantities of nitrogen in the casein and globulin and in the albumin precipitates from the total nitrogen.

TABLE V.  
*The total nitrogen contents and the amounts of nitrogen contained in the precipitates by Marshall's method and in the filtrates therefrom in human milk and cow's milk given as percentages of the whole milk.*

	TOTAL NITROGEN			NITROGEN IN MARSHALL'S PRECIPITATE			NITROGEN IN FILTRATE FROM MARSHALL'S PRECIPITATION		
	I	II	Average	I	II	Average	I	II	Average
<i>Human milk.</i>									
Sample No. 3 (colostrum).....	0.2885	0.3038	0.2962	0.2343	0.2253	0.2298	0.0724	0.0744	0.0734
Sample No. 2 (early milk).....	0.2181	0.2191	0.2186	0.1699	0.1728	0.1713	0.0496	0.0505	0.0500
<i>Cow's milk.</i>									
Samples of mixed milk	No. 2....	0.5121	0.5149	0.5135	0.4701	0.4839	0.0380	0.0385	0.0382
	No. 3....	0.5355	0.5029	0.5192	0.5035	0.5008	0.0406	0.0369	0.0388
	No. 4....	0.5129	0.5251	0.5190	0.5213	0.4983	0.0190	0.0226	0.0208
	No. 5....	0.5251	0.5019	0.5135	0.4732	0.4764	0.0350	0.0340	0.0345

*Experiments bearing on the amount and nature of the unknown material contained in milk as well as upon the protein content.*

Camerer and Söldner subtracted the sum of the quantities of lactose, fat, ash and citric acid<sup>12</sup> in their samples of milk from the amount of total solids, and called the remainder so obtained the quantity of the "protein plus unknown substances." They found that the nitrogen in cow's milk made up about 16 per cent of the quantity of "protein plus unknown substances," while that in human milk made up only from 11 to 12 per cent.<sup>13</sup> They then precipitated the protein from milk by alcohol according to Munk's method, and found that the dry ash-free protein of cow's milk contained 14.54 per cent of nitrogen; while that from human milk contained 13.64 per cent.<sup>14</sup> From all these results they concluded that human milk contains a considerable amount of unknown substances which contain little or no nitrogen.

Camerer and Söldner found, as have other investigators, that alcohol does not precipitate the nitrogenous substances from milk so completely as does tannic acid; further, the filtrate from the alcohol precipitation gives a positive reaction with the biuret test and with Millon's reagent. They have studied the non-fatty material which goes into solution in alcohol when the proteins are precipitated from milk by Munk's method and find that it is a soft, sticky material very slightly soluble in water but highly soluble in dilute alkali. Dilute hydrochloric acid gives a white flocculent precipitate from the alkaline solutions. The substance contained about 13 per cent of nitrogen, 0.2 per cent of phosphorus and 1 per cent of ash. From the high nitrogen and phosphorus contents Camerer and Söldner suspect that the material is altered casein.<sup>15</sup>

Meigs and his collaborators have gone over the ground covered by Camerer and Söldner on the protein and unknown substances in milk: Their most important results are given in Tables VI and VII.

<sup>12</sup> Camerer and Söldner: *Zeitschr. f. Biol.*, xxxvi, p. 278, 1898.

<sup>13</sup> Camerer and Söldner: *ibid.*, xxxiii, p. 549 and pp. 562-565, 1896.

<sup>14</sup> Camerer and Söldner: *ibid.*, xxxiii, p. 548, 1896; The alcohol precipitate from the cow's milk contained, on the average, 11.06 per cent of ash; that from the human milk, 6.29 per cent.

<sup>15</sup> Camerer and Söldner: *ibid.*, xxxiii, p. 561, 1896.



TABLE VI.

*The quantities of total solids and of "protein plus unknown substances" in human milk and cow's milk given as percentages of the weight of the whole milk; and the percentages of nitrogen which may be supposed to be contained in the "protein plus unknown substances."*

		TOTAL SOLIDS			PROTEIN PLUS UNKNOWN SUBSTANCES	PERCENT- AGE OF NITROGEN IN PROTEIN PLUS UNKNOWN SUB- STANCES*
		I	II	Average		
<i>Human milk.</i>						
5th to 9th day post partum...	Milk No. 4 . . . . .	11.118	11.128	11.123	1.911	11.73
	Milk No. 6 . . . . .	11.238	11.285	11.263	2.364	11.70
	Milk No. 7 . . . . .	11.002	11.064	11.033	1.584	12.35
	Milk No. 8 . . . . .	10.997	10.997	10.997	2.219	11.82
	Milk No. 10 . . . . .	12.446	12.416	12.431	2.415	11.78
Milk No. 9, 5th month post partum		12.670	12.660	12.665	1.274	12.63
Milk No. 5, collected near end of lactation . . . . .		9.604	9.569	9.586	1.221	16.05
<i>Cow's milk.</i>						
Samples of mixed milk from grade cows . . . . .	No. 7 . . . . .	13.320	13.471	13.395	3.769	14.07
	No. 8 . . . . .	13.437	13.484	13.460	3.744	14.73
	No. 11 . . . . .	13.195	13.188	13.191	3.640	15.36
	No. 15 . . . . .	13.343	13.313	13.328	3.249	15.58
	No. 16 . . . . .	12.459	12.503	12.481	3.066	16.23
No. 19 . . . . .		11.688	11.754	11.721	3.106	16.84
Samples of milk from single thoroughbred Guernsey cows . . . . .	No. 1 . . . . .	15.265	15.230	15.247	3.976	14.14
	No. 9 . . . . .	14.080	14.104	14.092	3.674	13.50
	No. 13 . . . . .	14.035	14.033	14.034	3.725	15.97

\* The average percentage of nitrogen in the "protein plus unknown substances" in samples 4, 6, 7, 8, 9 and 10 of human milk is 12 per cent. Sample 5 is omitted from this calculation, as the milk came from very near the end of lactation and was evidently abnormal. The average percentage of nitrogen in the "protein plus unknown substances" in the samples of cow's milk is 15.18 per cent.

Table VI gives the amount of total solids and of "protein plus unknown substances" in a number of samples of human milk and of cow's milk. The figures for the "protein plus unknown substances" were obtained in the same way as those of Camerer and Söldner. The average nitrogen contents of the "protein plus unknown substances" as calculated from our figures is 12 per cent in the case of human milk and 15.18 per cent in the case of cow's milk.

TABLE VII.

The dry weight of protein precipitated by alcohol from fat-free human milk and cow's milk given as percentages of the weight of the whole milk; the amount of nitrogen contained in the alcohol precipitate given as percentage of the weight of the whole milk; and the percentages of nitrogen contained in the dry alcohol precipitates.

DRY WEIGHTS OF ALCOHOL PRECIPITATES*		AMOUNTS OF NITROGEN CONTAINED IN ALCOHOL PRECIPITATES GIVEN AS PERCENTAGES OF WHOLE MILK			AMOUNTS OF NITROGEN CONTAINED IN ALCOHOL PRECIPITATES GIVEN AS PERCENTAGES OF DRY ASH-FREE PRECIPITATES		
		I	II	Average	I	II	Average
<i>Human milk.</i>							
Precipitated by 75	{ Milk No. 4...	1.064	1.052	1.058	0.1506	0.1530	0.1518
per cent alcohol	{ Milk No. 5...	0.911	0.809	0.890	0.1465	0.1408	0.1466
Precipitated by 86	{ Milk No. 8...	1.292	1.315	1.303	0.1666	0.1666	0.1666
per cent	{ Milk No. 9...	0.786	0.793	0.789	0.1049	0.1024	0.1036
	{ Milk No. 10...	1.440	1.428	1.434	0.1941	0.1970	0.1955
Precipitated by 90	{ Milk No. 7...	0.991	0.957	0.974	0.1474	0.1471	0.1472
per cent alcohol							15.37
							14.87
							15.11
							14.54
							16.89
							12.67
							12.78
							12.91
							13.13
							13.80
							13.64
							15.37
							14.87
							15.11
							14.54
							16.89
							12.67
							12.78
							12.91
							13.13
							13.80
							13.64
							15.37
							14.87
							15.11
							14.54
							16.89
							12.67
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							13.13
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							13.64
							15.37
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							12.78
							12.91
							13.13
							13.80
							13.64
							15.37
							14.87
							15.11
							14.54</

The dry weight of the material precipitated from samples of human milk and cow's milk by alcohol and the nitrogen content of this material are given in Table VII. The ash-free protein precipitated from human milk by alcohol contained on the average 13.80 per cent of nitrogen; while that from cow's milk contained 14.07 per cent.

The following is an account of the methods used by Meigs and his collaborators in this part of the investigation.

Samples of milk were placed in platinum dishes (without sea sand) and were kept in a water oven at about 98°<sup>16</sup> until ten hours after the visible fluid in them had disappeared. The residues were then placed in desiccators over sulphuric acid at room temperature until they reached constant weight, and this figure was taken as the weight of the total solids. The lactose retains its water of crystallization under these conditions of drying.<sup>17</sup>

The protein precipitated from milk by alcohol was determined as follows: The fat was removed from 10 cc. of milk by Meigs' method described on pages 151-153. After the removal of the fat had been completed the lower layer of the mixture (which contained the non-fatty constituents from 10 cc. of milk) was transferred to a 200 cc. beaker and evaporated on a water bath to a volume of about 10 cc.; 95 per cent alcohol was then added. The amount of alcohol added differed in different experiments; in some cases the mixture obtained by adding the alcohol contained 75 per cent of alcohol; in others, 86 per cent; and in still others, 90 per cent of alcohol. The mixture was allowed to stand five minutes and then filtered through a weighed porous alundum crucible by means of a suction pump and washed with 1 liter of boiling alcohol and subsequently with 500 cc. of ether. Before being washed, the protein was carefully broken up into small particles with a glass rod.

The contents of the crucible were dried first in a water oven at 98° and then in a desiccator over sulphuric acid at room temperature until they reached constant weight. They were then weighed, and their content of nitrogen was afterwards determined.

The filtrates from the milk treated with alcohol as described above were studied in various ways. It was found that if these filtrates were evaporated to small volume and then treated with a considerable quantity of water, there was precipitated from them a material which resembled that described by Camerer and Söldner (see page 158). Marsh determined the solubilities of this material

<sup>16</sup> Temperatures are throughout this article expressed in terms of the centigrade scale.

<sup>17</sup> Camerer and Söldner: *Zeitschr. f. Biol.*, xxxiii, pp. 538-540, 1896.

and the nitrogen content. He found that all of it was soluble in alcohol and only a portion in ether. The ether-insoluble portion of the material is insoluble in water and in acids; soluble in alcohol, chloroform and alkalies. It gives a positive reaction to both Millon's test and the biuret. It contains 13.8 per cent of nitrogen. On being ignited, it leaves a little ash which contains phosphorus. It can be obtained in larger quantities from human milk than from cow's milk.



FIG. 2. CRYSTALS OF SUBSTANCE OF UNKNOWN COMPOSITION FROM HUMAN MILK.

It has been found possible to obtain two kinds of crystals from the alcohol filtrate of human milk treated as described on page 161. Of these one appears as long glistening prisms. These crystals are soft and represent, in all probability, a minute remnant of fat, which had not been removed by Meigs' method of fat extraction. The appearance of the other kind of crystal is shown in Figure 2, which is reproduced from a photograph.

It was afterward found that the crystals shown in Figure 2 could most easily be obtained as follows:

The fat was removed from samples of milk by Meigs' method described on pages 151-153; the fat-free or nearly fat-free lower layer was then shaken up with ether; the mixture allowed to stand; and the upper layer, consisting of a mixture of alcohol and ether, was drawn off. This was evaporated over boiling water to a volume of about 20 cc. and then allowed to evaporate spontaneously. The crystals usually made their appearance before the supernatant fluid had entirely evaporated. The production of the crystals has, however, been rather capricious; no method has yet been devised by which they could be obtained with certainty.

We have submitted these crystals to Dr. A. P. Brown, Professor of Geology and Mineralogy in the University of Pennsylvania, for examination and have obtained the following report:

The crystals belong to the Tetragonal System.

Their axial ratio is  $a : c = 1 : 2.46$ .

Form observed: The unit pyramid was the only form present.

Angles: The plane angle of the face of the unit pyramid, between the pole edges  $111 - 111 \wedge 111 - 111 = 35^\circ 20'$ ; also the edge angle over the pole, edges  $111 - 111 \wedge 111 - 111 = 44^\circ 20'$  (calculated  $44^\circ 6'$ ).

Habit of crystals: The unit pyramid is alone developed; sometimes symmetrically, more often distorted by contact with the surface upon which it rests, giving the appearance at first sight of the unit pyramid combined with the basal pinacoid (001) and with the unit prism (110), but these forms are not seen. No twins were observed, but parallel growths of the unit pyramids were fairly common. The crystals are brittle; no cleavage was observed.

The refractive index is high and the double refraction is very strong. Examined in polarized light, the extinction is straight in all ordinary positions. The greater refractive index is for light vibrating parallel to the crystal axis,  $c$ ; hence  $\epsilon > \omega$ , or the crystal is optically positive (+). On examining the crystal in the direction of the vertical axis,  $c$ , in convergent polarized light, the uniaxial interference figure is observed, thus confirming the tetragonal character.

As a check on the angles, the angle over the pole was measured, angle  $111 \wedge 111 = 32^\circ 40'$  as against  $32^\circ 4'$  calculated. The profile view of the crystal lying on the pole edge gave an edge angle over the pole of about  $54^\circ$ .

These crystals are fairly soluble in ether, somewhat soluble in 95 per cent alcohol, and nearly insoluble in water, though treatment with water alters them, rendering them opaque and in time destroying their crystalline form. They dissolve very slowly in acetone. They have been submitted to Lassaigne's test for nitrogen, and thereby shown to be free from nitrogen; but the appli-

cation of the plumbic acetate test<sup>18</sup> shows that they contain a considerable amount of sulphur.

They represent a substance, which, so far as we know, has not been heretofore described as a component of milk.

The immediately preceding paragraphs may be summed up by saying that the results of Meigs and his collaborators on the protein and unknown substances of milk are throughout in close agreement with those of Camerer and Söldner. Both sets of observers find that the total nitrogen in human milk makes up a decidedly smaller proportion of the "protein plus unknown substances" than it does in the case of cow's milk; both sets of observers find that the materials precipitated from the two kinds of milk by alcohol have about the nitrogen content which is usual for protein; finally, both sets of observers obtained similar materials from the alcohol filtrates. This agreement is made all the more striking by the fact that neither Meigs nor any of his collaborators was familiar with the work of Camerer and Söldner at the time when their experiments were carried out. They obtained their results quite independently. We are inclined, therefore, to regard it as established, or at least very highly probable, that human milk contains a considerable amount of unknown material, which has either a low nitrogen content or else none at all of that element.

What becomes of this material when milk is subjected to analysis? It has been shown above that unknown substances can be extracted from milk by means of alcohol, and we shall consider very briefly the question whether the material of the alcohol extract is to any extent identical with the "x material," the existence of which in human milk can be inferred from the work of Camerer and Söldner and of Meigs and his collaborators.

Camerer and Söldner advance the hypothesis that the material of the alcohol extract is "altered casein." We think this improbable for the following reason. Cow's milk contains about three times as much casein as human milk. If, therefore, alcohol alters casein so that a part of it becomes alcohol-soluble, more of this altered casein should appear in the alcohol filtrate from

<sup>18</sup> Hawk: *Practical Physiological Chemistry*, 4th edition, Philadelphia, 1912, pp. 108, 109.

cow's milk than in that from human milk. But just the opposite is the case.

We have endeavored to get an approximate idea of the total amount of unknown material in the alcohol extract from cow's milk and human milk. Two samples of human milk and three samples of cow's milk were freed from fat by Meigs' method, and the protein was precipitated from the fat-free residue by 86 per cent alcohol.

The filtrates from the 86 per cent alcohol protein precipitations were then evaporated to dryness and the weights of the ash-free residues were determined. These residues contained all the lactose from the milk plus the unknown alcohol-soluble material. In other portions of the same samples of milk the lactose was determined by the Fehling method—see Table III. Subtracting the amounts of lactose from those of the ash-free residues in the five samples of milk gives the following results:

		<i>Percentage of ash-free alcohol residue</i>		<i>Percentage of lactose</i>		<i>Percentage of unknown alco- hol-soluble material</i>
Human milk..	{ No. 10.....	7.952	—	6.574	=	1.378
	{ No. 9.....	7.575	—	7.080	=	0.495
Cow's milk...	{ No. 16.....	5.104	—	4.713	=	0.391
	{ No. 19.....	5.068	—	4.482	=	0.586
	{ No. 13.....	4.935	—	4.748	=	0.187

The following is another method by which an approximate idea of the amount of unknown alcohol-soluble material in milk may be gained. In the samples of human milk Nos. 4, 5, 7, 8, 9 and 10 the weights of the material precipitated by alcohol have been determined (Table VII) and may be compared with the quantities of "protein plus unknown substances" found in Table VI. It will be seen that the quantities of the latter are always considerably larger than those of the former; the results obtained by subtracting the quantities of ash-free alcohol precipitates from those of the "protein plus unknown substances" are as follows: Milk No. 4, 0.853; No. 7, 0.610; No. 8, 0.916; No. 10, 0.981; No. 9, 0.485; No. 5, 0.331. The same data for the samples of cow's milk, Nos. 7, 8, 9, 13 and 19 are given in Tables VI

and VII; and the results of similar calculations are as follows: Milk No. 7, 0.357; No. 8, 0.334; No. 9, 0.281; No. 19, 0.076.<sup>19</sup>

The figures which have just been given to represent the amounts of the unknown alcohol-soluble material in human milk at different periods of lactation correspond very fairly with the figures for the amounts of the  $x$  material at the corresponding periods of lactation as calculated from the data of Camerer and Söldner and from those of Meigs and his collaborators. It seems probable, therefore, that the alcohol-soluble material is to a considerable extent identical with the  $x$  material. That it is not wholly identical with the  $x$  material is evident from general considerations as well as from its high nitrogen content. Milk contains such bodies as urea, ammonia,<sup>20</sup> and purine bases<sup>21</sup> which would not be precipitated by alcohol; and the nitrogen in these would account for a considerable part of the nitrogen in the alcohol-soluble material.

*The known alcohol-soluble substances in milk.*

Koch and Woods<sup>22</sup> find 0.036 to 0.049 per cent lecithin and 0.027 to 0.045 per cent cephalin in cow's milk—0.072 to 0.086 per cent total "lecithans."<sup>23</sup> For human milk the figures of these investigators are lecithin, 0.041 per cent; cephalin, 0.037 per cent; total lecithans, 0.078 per cent.

Raudnitz<sup>24</sup> gives a review of previous determinations of cholesterol<sup>25</sup> in milk. Tolmatscheff found 0.0252 to 0.0385 per cent of this substance in human milk; Schmidt-Mülheim was able to demonstrate its presence in cow's milk. Marsh determined the amount of cholesterol in cow's milk according to the method of Ritter<sup>26</sup> and found 0.021 per cent.

<sup>19</sup> In the case of sample No. 13 of cow's milk there was some error in analysis so that the weight of the material precipitated by alcohol appeared to be slightly greater than that of the "protein plus unknown substances."

<sup>20</sup> Camerer and Söldner: *Zeitschr. f. Biol.*, xxxvi, p. 299, 1898.

<sup>21</sup> Raudnitz: *Ergeb. d. Physiol.*, 1903, ii, p. 257.

<sup>22</sup> Koch and Woods: *This Journal*, i, p. 211, 1906.

<sup>23</sup> By "lecithans" Koch and Woods mean the phosphorus-containing lipoids.

<sup>24</sup> Raudnitz: *Ergeb. d. Physiol.*, 1903, ii, p. 264.

<sup>25</sup> Raudnitz uses the older term, "cholesterin."

<sup>26</sup> Ritter: *Zeitschr. f. physiol. Chem.*, xxxiv, p. 430, 1901-02.



The substances which have been spoken of above—lecithin, kephalin, cholesterol, urea, ammonia and the purine bases—would account for less than one-fifth of the unknown material in the alcohol extract of human milk.

It is realized that the data given above and bearing on the quantity of the material in the alcohol extract of milk are very incomplete. But they do indicate that human milk contains considerably more non-fatty, alcohol-soluble material than cow's milk; and it has been thought worth while to present them in view of recent interesting work on the importance of unknown substances in milk as accessory factors in diet. Stepp,<sup>27</sup> for instance, has found that mice always die in a few weeks when given food materials which have been fully extracted with alcohol and ether, and that the diet may be rendered again capable of sustaining life by the addition of the material from the alcohol-ether extract of skimmed milk. Stepp has been able to show that the material necessary to maintain life is not either fat, cholesterol, lecithin or salts.

#### GENERAL CONCLUSIONS.

Human milk differs from cow's milk in three important ways. It contains considerably more lactose than cow's milk, and more substances of unknown nature which contain little or no nitrogen; it contains very much less protein than cow's milk. The composition of milk varies more or less regularly with the progress of lactation so that average figures for its composition are not very satisfactory. The following, however, may be taken as the limits of normal variation of the constituents of the two kinds of milk from the beginning of the second month of lactation onward, the figures representing percentages of whole milk:

	FAT	LACTOSE	PROTEIN
Human milk.....	2-4	6-7.5	0.7-1.5
Cow's milk.....	2-4	3.5-5	2.5-4

Both kinds of milk contain substances which are important constituents of diet, which are soluble in alcohol and ether, which

<sup>27</sup> Stepp: *Zeitschr. f. Biol.*, lvii (N. F. xxxix), p. 135, 1911.

contain little or no nitrogen, but of which the chemical nature is still unknown. These substances are most plentiful in early human milk and diminish in amount with the progress of lactation. Early human milk contains about 1 per cent of these unknown substances; milk from the middle period of lactation about 0.5 per cent. Cow's milk from the middle period of lactation contains about 0.3 per cent of the unknown substances.

# THE INFLUENCE OF THE ADMINISTRATION OF CREATINE AND CREATININE ON THE CREATINE CONTENT OF MUSCLE.

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With the appearance of an accurate method of estimating creatinine, the story of the origin of this interesting urinary constituent has been found to be less simple than was formerly supposed. Without doubt, the information which has been brought to light with its aid has been very important, although in many instances this has been contrary to the previously accepted views, and difficult of satisfactory explanation. That the creatinine of the urine has its origin in the creatine of the muscle seems obvious on *a priori* grounds, but the satisfactory demonstration of this has been beset with unforeseen difficulties.<sup>1</sup> Folin,<sup>2</sup> with the aid of his colorimetric method, was the first to point out that the *quantitative* conversion of creatine to creatinine or creatinine to creatine *in vitro* was far more difficult than previous statements would lead one to believe. That the body was even less able to bring about a quantitative conversion of creatine to creatinine was shown by his feeding experiments on man. In fact he was unable to adduce any evidence of a conversion of the administered creatine to creatinine. Further experimental data bearing on the fate of administered creatine and creatinine have been presented by Klercker,<sup>3</sup> Wolf and Shaffer,<sup>4</sup> van Hoogenhuyze and Verploegh,<sup>5</sup>

<sup>1</sup> For an interesting critical review of the general subject of creatine-creatinine metabolism, reference may be made to the paper of Riesser: *Zeitschr. f. physiol. Chem.*, lxxxvi, p. 415, 1913.

<sup>2</sup> Folin: *Hammarsten's Festschrift*, III, 1906.

<sup>3</sup> Klercker: *Beitr. z. chem Physiol. u. Path.*, viii, p. 59, 1906; *Biochem. Zeitschr.*, iii, p. 45, 1907.

<sup>4</sup> Wolf and Shaffer: this *Journal*, iv, p. 439, 1908.

<sup>5</sup> van Hoogenhuyze and Verploegh: *Zeitschr. f. physiol. Chem.*, lvii, p. 161, 1908.

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Lefmann,<sup>6</sup> Plimmer, Dick and Lieb,<sup>7</sup> Pekelharing and van Hoogenhuyze,<sup>8</sup> Foster and Fisher,<sup>9</sup> Towles and Voegtlin,<sup>10</sup> Folin and Denis,<sup>11</sup> and Kraus.<sup>12</sup> It has been observed that when creatinine is administered to man or animals, either *per os* or parenterally, about 80 per cent reappears in the urine during the succeeding twenty-four hours. After the administration of creatine, the excretion of creatinine has been found to be only slightly increased, if at all. When given in comparatively large amounts a considerable portion may reappear in the urine as such, though with small amounts it does not reappear. In contradistinction to creatinine, Folin has viewed the creatine under these conditions not as a waste product but as a food.

Since the administered creatine cannot be accounted for by the creatine (and creatinine) of the urine, it seems logical to assume that this remaining portion is either metabolized or stored up in the muscle. This last possibility was considered by Mellanby<sup>13</sup> in experiments on rabbits and chickens. He concludes, "Creatine and creatinine feeding has no effect upon the creatine content of muscle after the muscle has reached a certain saturation point. There is some evidence of an increase in muscle creatine at an early stage of life on feeding with creatine and possibly with creatinine." In an endeavor to throw light upon the origin of creatine, Riesser,<sup>14</sup> in his recent studies, observed a slight increase in the concentration of muscle creatine after the administration of betaine and choline.

The possibility that the creatine, which does not reappear in the urine after its introduction into the body, is deposited in the muscle tissue, has not been answered, and it was primarily for this reason that the present experiments were undertaken. In his experiments Mellanby took no account of the portion of the ad-

<sup>6</sup> Lefmann: *Zeitschr. f. physiol. Chem.*, lvii, p. 476, 1908.

<sup>7</sup> Plimmer, Dick and Lieb: *Journ. of Physiol.*, xxxix, p. 98, 1909.

<sup>8</sup> Pekelharing and van Hoogenhuyze: *Zeitschr. f. physiol. Chem.*, lxi, p. 395, 1910.

<sup>9</sup> Foster and Fisher: *this Journal*, ix, p. 359, 1911.

<sup>10</sup> Towles and Voegtlin: *ibid.*, x, p. 479, 1912.

<sup>11</sup> Folin and Denis: *ibid.*, xii, p. 148, 1912.

<sup>12</sup> Kraus: *Arch. of Int. Med.*, xi, p. 613, 1913.

<sup>13</sup> Mellanby: *Journ. of Physiol.*, xxxvi, p. 470, 1908.

<sup>14</sup> *Loc. cit.*

ministered creatine or creatinine that might be lost in the urine. Obviously, this is a factor of importance in the interpretation of the content of muscle creatine. Such analyses were included in the present experiments, in order that we might ascertain in so far as possible the fate of *all* the administered creatine and creatinine.

#### METHODS.

The general analytical procedures employed in the analysis of tissue and urine were those previously described.<sup>15</sup> The creatine used in the first experiment (rabbit 47) was prepared from rabbit muscle. In the succeeding experiments, however, the creatine and creatinine were prepared from urine according to unpublished directions supplied us by Prof. S. R. Benedict. With these methods we have prepared about 100 grams of creatine and 10 grams of creatinine, both of the highest degree of purity. Our thanks are due to Professor Benedict for furnishing us with the details of these admirable methods. In all experiments the creatine and creatinine were administered subcutaneously in 1 per cent aqueous solution between the shoulder blades with a 10 cc. Luer glass syringe. In every case the strength of the solution employed was ascertained colorimetrically, although in several experiments this was checked by employing carefully dehydrated preparations, which could be accurately weighed. The time of administration was generally from 2-5 hours after the end of the previous twenty-four-hour period. The injections extended over a period of 5-13 consecutive days. It was believed that in this way the maximum retention of creatine in the muscle would be observed, and further that the average daily composition of the urine during this interval would furnish a very reliable comparison with that of the control period. There appeared to be a lag in the elimination of urine in one or two cases, notably in rabbit 71, although it is improbable that this could have had an important influence upon the average data of a period of several days. Nitrogen determinations were made on the urines of all rabbits with the exception of rabbits 71, 72, 73 and 74. The daily variations were so slight as to fall within the limits of error; and as they are without significance for our present purposes, they have not been included in this paper.

<sup>15</sup> Myers and Fine: this *Journal*, xiv, p. 9, 1913.

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### EXPERIMENTAL PART AND DISCUSSION.

Eleven injection experiments with creatine and creatinine are reported upon rabbits, eight with creatine and three with creatinine. Two of the eight experiments with creatine (numbers 71 and 72) did not include estimations of the creatine of the muscle, while one experiment, number 57, was carried out on a rabbit fed upon a practically pure carbohydrate diet.<sup>16</sup> The important results of these experiments are summarized in Tables I and II, the experimental details being recorded in the Tables III–XII appended to this paper. The creatine employed in experiments 47, 49, 56, 59 and 57 was tested qualitatively for creatinine and failed to give an appreciable reaction. Following the period of injection, the creatinine excretion was found to be slightly increased, the equivalent of a conversion of about 3 per cent of the administered creatine. On this account, three additional experiments, 71, 72, and 73, were conducted employing creatine, the purity of which had been quantitatively ascertained. Four colorimetric tests were made with this preparation during the course of the experiments, employing in each case 0.1-gram portions. The colorimetric readings indicated that the possible contamination of creatinine was not over 0.15 to 0.20 per cent, an amount insignificant in this connection. It is interesting to note that even with this very pure preparation of creatine there was an increased elimination of creatinine, if anything greater than that observed in the earlier trials.

The data on the influence of the administration of creatine and creatinine on the creatine content of the muscle are summarized in Table I. The results for the nitrogen and moisture content of the muscle are very uniform and all fall within normal limits. The figures for the creatine content are, however, uniformly above the usual normal figure of 0.52 per cent, if we except carbohydrate rabbit 57; and even here the creatine content is considerably higher than in other animals fed carbohydrate but no creatine.<sup>17</sup> The increase after the creatine injections in the first five experiments amounts to about 5 per cent.<sup>18</sup> It is true that this increase is slight

<sup>16</sup> See Myers and Fine: *this Journal*, xv, p. 305, 1913.

<sup>17</sup> *Loc. cit.*

<sup>18</sup> This is the percentage increase over the normal 0.522 per cent. The absolute amount of creatine retained by the body is calculated by mul-

TABLE I.

*The creatine content of rabbit muscle as influenced by the subcutaneous administration of creatine and creatinine.*

ANIMAL	BODY WEIGHT	CREATINE CONTENT OF BODY		COMPOSITION OF MUSCLE			LENGTH OF TIME AFTER LAST INJECTION BEFORE KILLING ANIMAL
		Calculated Body Wt. $\times 0.182^*$	Found	Nitrogen	Molsture	Creatine	

*Experiments with creatine.*

	kgms.	grams	grams	per cent	per cent	per cent	days
47	1.65	3.00	3.02	3.71	76.1	0.544	3
49	1.72	3.13	3.47	3.61	75.9	0.546	3
56	1.81	3.29	3.70	3.35	77.1	0.559	3
59	2.00	3.64	3.90	3.52	76.7	0.553	4
73	1.66	3.02	3.04	3.63	75.3	0.540	1
57†	1.98-1.22	3.60	2.18	3.55	76.2	0.482	1

*Experiments with creatinine.*

	kgms.	grams	grams	per cent	per cent	per cent	days
58	1.68	3.06	3.49	3.55	76.3	0.540	3
62	1.70	3.02	3.09	3.45	76.4	0.566	4
74	1.93	3.51	4.04	3.59	76.0	0.566	1

\* Calculated from average data in Table VII of a previous paper, this *Journal*, xiv, p. 23, 1913.

† See Myers and Fine: *ibid.*, xv, p. 305, 1913.

and we would attach greater significance to the fact that a large amount of creatine was not stored up in the muscle than to the fact that a small amount was retained. This slight increase we believe, however, to be a little beyond the limits of error of the method when carefully carried out. Many colorimetric readings were made and compared with readings from control animals. The controls were found under the conditions of our experiments to give the usual readings of about 9.0 mm., whereas the samples from the animals to which the creatine and creatinine had been administered gave readings varying between 8.3 and 8.7 mm. As a further control, it was found that creatine added to muscle before extraction could be quantitatively recovered under the same conditions. The increase in the creatine content of the

tipling the increase per gram of muscle by the total muscle tissue of the body. The latter is estimated by dividing the total body creatine by the percentage found.

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muscle is, if anything, more pronounced after the administration of creatinine than of creatine. It would not be expected that creatinine would be retained by the muscle unchanged, and in the case of rabbit 74 a creatinine estimation<sup>19</sup> on the fresh muscle showed the presence of about 9 mgms. of creatinine per 100 grams of muscle, an amount not far from the normal. Rabbit 73, to which creatine had been given, contained 7 mgms. creatinine per 100 grams of muscle. It seems evident from the above trials that the creatine content of the muscle tissue can be raised slightly above the ordinary level by the administration of either creatine or creatinine. That creatinine exerts this influence is in harmony with the view that the reaction between these two substances is reversible. This is further borne out by the observations of other workers of an excretion of creatine following the administration of creatinine. The increase in the concentration of muscle creatine in rabbits, which Riesser<sup>20</sup> observed after the injection of betaine and choline is quite similar to that observed in our own experiments; and it is further of interest that in his six control animals he obtained figures identical with those which we have observed, viz., 0.52 per cent.<sup>21</sup>

Further evidence of a small retention of creatine is found in the comparison of the total content of creatine in the body calculated from the body weight with that actually found to be present, the latter always being the greater. The error incident to this comparison is, of course, considerable, but, nevertheless, the fact that the figures based upon actual determinations are uniformly higher than those calculated lends support to the above view. It would seem, then, that by the administration of either creatine or creatinine the concentration of muscle creatine may be raised about 5 or 6 per cent above the values ordinarily obtained. That the extra creatine is quite firmly held is indicated by the fact that the concentration of the creatine in the muscle is practically the same whether the rabbit is killed one day or four days after the last injection.

The various factors concerned in the fate of creatine and

<sup>19</sup> The method employed will be described in a subsequent paper.

<sup>20</sup> *Loc. cit.*

<sup>21</sup> Myers and Fine: this *Journal*, xiv, p. 14, 1913.



TABLE II.

*The fate of creatine and creatinine when administered subcutaneously in the rabbit.*

ANIMAL	BODY WEIGHT	TOTAL AMOUNT OF CREATINE OR CREATININE INJECTED	PERIOD OF INJECTION	CREATINE OR CREATININE INJECTED PER KGM. PER DAY	FATE OF ADMINISTERED MATERIAL			
					Eliminated as creatine in urine	Elim. as creatinine in urine	Retained as creatine in muscle	Unaccounted for

<i>Experiments with creatine.</i>								
	kgms.	grams	days	mgms.	per cent	per cent	per cent	per cent
47	1.65	0.95	5	115	60	4	13	23
49	1.72	1.02	12	49	26	7	15	52
56	1.81	1.06	10	59	32	2	23	43
59	2.00	1.22	9	85	67	1	18	14
71	2.55	0.90	6	59	53	10		
72	2.45	0.90	6	61	47	14		
73	1.66	0.70	7	60	81	7	14	0
57	1.98-1.22	1.09	13	52	58	2		

<i>Experiments with creatinine.</i>								
	kgms.	grams	days	mgms.	per cent	per cent	per cent	per cent
58	1.68	0.75	9	50	0	82	13	5
62	1.70	0.70	6	69	0	80	28	0
74	1.93	0.90	6	78	0	77	26	0

creatinine when introduced into the body are recorded in Table II. When creatine is administered subcutaneously to rabbits in amounts varying between 50 and 100 mgms. per kilogram of body weight per day, a considerable portion (25-80 per cent) reappears in the urine unchanged. In most of the trials about 3 to 4 per cent appeared in the urine as creatinine, although the amount excreted in this form may be considerably greater, as in rabbits 71 and 72. A possible explanation for these high results of 10 and 14 per cent may be found in the fact that these animals were very large and had "creatinine coefficients" of 16 and 17 respectively. As we have previously pointed out,<sup>22</sup> the average creatinine coefficient for the rabbit is 14, and these higher coefficients possibly indicate a greater efficiency on the part of these animals in the conversion of creatine to creatinine.

<sup>22</sup> Myers and Fine: this *Journal*, xiv, p. 19, 1913.

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Van Hoogenhuyze and Verploegh were apparently the first to call attention to the slightly increased excretion of creatinine after the administration of creatine, an observation confirmed by Pekelharing and van Hoogenhuyze, Towles and Voegtlin, S. R. Benedict<sup>23</sup> and the present writers. Although creatine and creatinine may not have the very simple metabolic relationship formerly supposed, it is not quite obvious how Klercker and Lefmann from their data draw the conclusion that exogenous creatine is not transformed to creatinine at least in small part.

In a previous communication,<sup>24</sup> attention was called to the significant fact that the percentage of administered creatine which was converted to creatinine was not widely different from the relationship existing between the daily creatinine of the urine and the total body creatine. It was further suggested that, since creatine is held in such a loose state of combination in the muscle, it is not illogical to believe that it experienced the same fate as the administered creatine.

Under the conditions of our experiments, it appears that about 15 per cent of the injected creatine may be stored in the muscle. The portion of the creatine remaining unaccounted for seems to be chiefly dependent upon the amount of the creatine administered, or, in other words, upon the opportunity given the body to oxidize it. The experimental data in the case of creatinine seem to indicate that the 20 per cent which is not excreted in the urine may be completely stored up in the muscle as creatine.

### CONCLUSIONS AND SUMMARY.

The subcutaneous administration of creatine to rabbits appears to cause a small increase in the creatine content of the muscle, about 5 per cent in five experiments. This is quite insufficient, however, to account for the creatine which does not reappear in the urine.

The administration of creatinine appears to exert a similar influence upon the creatine content of the muscle. In three experiments the creatine content was found to be about 6 per cent above the normal, an amount sufficient to account for the creatinine

<sup>23</sup> Private communication.

<sup>24</sup> Myers and Fine: this *Journal*, xv, p. 304, 1913.

not eliminated by the kidneys. This apparent increase in the creatine content of the muscle was not due to a retention of the creatinine unchanged.

Of the creatine administered in our experiments, 25–80 per cent—the quantity depending upon the amount injected—reappeared in the urine unchanged, while from 2–10 per cent was eliminated in the form of creatinine. We are inclined to attach considerable significance to this small conversion of creatine to creatinine, as throwing light upon the relationship of these two substances in metabolism.

When creatinine was administered 77–82 (average 80) per cent reappeared in the urine. No elimination of creatine was detected.

TABLE III.  
*Rabbit 47—Injection of creatine.*

DAY	BODY WEIGHT	URINE			CREATINE INJECTED	CREATINE RECOVERED IN URINE
		Volume	Creatinine	Creatine		
	<i>kgms.</i>	<i>cc.</i>	<i>mgms.</i>	<i>mgms.</i>	<i>mgms.</i>	<i>per cent</i>
1	1.60	280	57	0		
2	1.61	270	58	0		
3	1.65	230	57	0		
4	1.67	235	58	0		
5	1.69	250	54	0		
6	1.68	210	53	0		
7	1.63	285	60	0		
8	1.63	260	60	0		
Average.....			57			
9	1.65	260	60	244	328	74
10	1.66	250	66	237	306	77
11	1.68	285	67	35	131	27
12	1.65	225	70	26	109	24
13	1.67	230	61	30	77	39
Average.....			65	114	190	60
14	1.66	235	68	0		
15	1.69	260	61	0		

Female albino, received 350 grams carrots daily.

On days 9, 10, 11, the creatine was given in 3, 4 and 2 doses respectively, in the course of 4 to 6 hrs.

Skinned and eviscerated carcass—870 grams.

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TABLE VI.  
*Rabbit 59—Injection of creatine.*

DAYS	BODY WEIGHT	URINE			CREATINE INJECTED	CREATINE RECOVERED IN URINE
		Volume	Creatinine	Creatine		
	<i>kgms.</i>	<i>cc.</i>	<i>mgms.</i>	<i>mgms.</i>	<i>mgms.</i>	<i>per cent</i>
1	2.07	295	80	0		
2	2.07	190	78	0		
3	2.12	160	72	0		
4	2.12	240	72	0		
5	2.07	200	78	0		
Average .....			76			
6	2.10	265	90	38	90	52
7	2.04	250	74	39	90	
8	2.01	280	69	62	90	
9	2.00	305	80	88	135	67
10	2.00	295	68	95	135	
11	1.98	185	70	94	135	
12	2.06	280	81	133	181	75
13	2.00	350	80	146	181	
14	1.98	330	77	128	181	
Average .....			77	91	135	67
15	1.98	335	71	0		
16	1.95	315	74	0		
17	1.95	315	80	0		
18	1.95	280	67	0		

Black female, first seven days fed 350 grams carrots and 400 grams on remaining days of experiment.

Skinned and eviscerated carcass—1072 grams.

TABLE VII.  
*Injection of creatine.*

DAY	BODY WEIGHT	URINE			CREATINE INJECTED	CREATINE RECOVERED IN URINE
		Volume	Creatinine	Creatine		

*Rabbit 71.*

	kgms.	cc.	mgms.	mgms.	mgms.	per cent
1	2.61	180	108	0		
2	2.58	85	109	0		
3	2.56	115	109	0		
4	2.63	125	108	0		
5	2.63	150	107	0		
Average .....			108	0		
6	2.62	185	133	50	100	34
7	2.57	100	102	30	100	
8	2.57	80	108	23	100	
9	2.55	175	123	135	200	63
10	2.51	200	131	125	200	
11	2.52	140	107	110	200	
Average .....			117	79	150	53
12	2.50	180	108	7		
13	2.50	205	129	0		

*Rabbit 72.*

1	2.43	230	111	0		
2	2.44	50	108	0		
3	2.48	90	111	0		
4	2.48	190	112	0		
5	2.45	270	118	0		
Average .....			112	0		
6	2.45	240	128	32	100	30
7	2.43	275	141	20	100	
8	2.40	220	131	38	100	
9	2.43	250	122	116	200	56
10	2.40	290	133	103	200	
11	2.48	230	128	118	200	
Average .....			130	71	150	47
12	2.40	255	122	0		
13	2.40	270	119	0		

Both animals were males. Rabbit 71 ate 180-350 grams carrots daily; rabbit 72, 350 grams carrots daily.

On days 9, 10, 11 in Both experiments the creatine was given in two doses at intervals of 2-4 hours.

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TABLE VIII.  
*Rabbit 73—Injection of creatine.*

DAY	BODY WEIGHT	URINE			CREATINE INJECTED	CREATINE RECOVERED IN URINE
		Volume	Creatinine	Creatine		
	<i>kgs.</i>	<i>cc.</i>	<i>mgms.</i>	<i>mgms.</i>	<i>mgms.</i>	<i>per cent</i>
1	1.66	125	61	0		
2	1.65	110	61	0		
3	1.61	40	56	6		
4	1.67	80	60	10		
5	1.68	125	61	7		
Average .....			60	5		
6	1.65	180	67	74	100	
7	1.65	180	75	120	100	
8	1.65	150	65	101	100	
9	1.66	200	66	100	100	
10	1.67	235	62	85	100	
11	1.67	230	66	65	100	
12	1.66	220	61	56	100	
Average .....			66	86	100	81

Female albino, ate 140-350 grams carrots daily.

The elimination of small amounts of creatine in the first period suggests the possibility that growth had not been completed.

Skinned and eviscerated carcass—847 grams.

TABLE IX.

*Rabbit 57—Carbohydrate feeding; Creatine injection.*

DAYS	BODY WEIGHT	DIET		DAILY OR AVERAGE DAILY URINARY DATA			CREATINE INJECTED	CREATINE RECOVERED IN URINE
		Sucrose	Water	Total N	Creatinine	Creatine		
	kgms.	grams	cc.	gram	mgms.	mgms.	mgms.	per cent
1-7	1.92	22	80	0.44	63	8		
8-12	1.57	20	60	0.41	69	7		
13	1.39	20	70	0.23	68	40	90	33
14		20	70				90	
15		20	70				90	
16		20	60				90	
17		20	50				90	
18		20	50				90	
19		20	50				90	
20	1.34	20	50	0.30	76	56	90	
21		20	50	0.20	81	65	90	
22		20	50	0.22	56	39	90	
23	1.38	20	50	0.40	87	127	90	
20-23 (Average)					75	72	90	60
24		20	50	0.29	68	38	0	
25		20	50	0.26	65	20	0	
26		20	50	0.22	51	13	0	
24-26 (Average)					61	24		
27	1.24	20	50	0.30	66	67	90	
28-29 (Average)	1.24	20	50	0.16	42	51	45 (90)	
27-29 (Average)					50	56	60	60

White and black female. From 12th to 17th day diarrhoea was present although by frequently compressing the bladder, good urine samples were obtained. After the 17th day no diarrhoea was observed. On the 29th day, the animal still appeared to be in good condition, but it was thought best to kill it so as to avoid the acute changes immediately preceding death.

Skinned and eviscerated carcass—687 grams.

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TABLE X.  
*Rabbit 58—Injection of creatinine.*

DAYS	BODY WEIGHT	URINE			CREATININE INJECTED	CREATININE RECOVERED IN URINE
		Volume	Creatinine	Creatine		
	<i>kgms.</i>	<i>cc.</i>	<i>mgms.</i>	<i>mgms.</i>	<i>mgms.</i>	<i>per cent</i>
1	1.79	355	68	0		
2	1.76	310	66	0		
3		250	67	0		
4	1.76	335	69	0		
5	1.71	290	67	0		
6	1.70	315	69	0		
7	1.79	260	64	0		
Average.....			67			
8	1.68	80	134	0	83	
9	1.58	275	131	0	83	
10	1.65	275	130	0	83	
11	1.68	290	126	0	83	
12	1.67	325	142	0	83	
13	1.67	220	107	0	83	
14	1.68	295	162	0	83	
15	1.66	265	126	0	100	
16	1.68	325	147	0	75	
Average.....			134		84	82
17	1.65	250	54	0		
18	1.68	265	64	0		
19	1.68	260	65	0		

Brown male, ate 350 grams carrots daily. On the 8th day, through an oversight, the animal was not fed, which accounts for the low volume of urine on this day.



TABLE XI.  
*Rabbit 62—Injection of creatinine.*

DAY	BODY WEIGHT	URINE			CREATININE INJECTED	CREATININE RECOVERED IN URINE
		Volume	Creatinine	Creatine		
	<i>kgms.</i>	<i>cc.</i>	<i>mgms.</i>	<i>mgms.</i>	<i>mgms.</i>	<i>per cent</i>
1	1.70	240	64	0		
2	1.73	230	62	0		
3	1.79	240	66	0		
4	1.82	260	63	0		
Average.....			64			
5	1.85	320	146	0	83	84
6	1.80	340	130	0	83	
7	1.66	250	137	0	83	
8	1.66	255	157	0	124	
9	1.66	260	184	0	165	75
10	1.66	305	192	0	165	
Average.....			159		117	80
11	1.64	280	57	0		
12	1.65	230	59	0		
13	1.66	265	59	0		
14	1.65	195	59	0		

Black and white female, ate 350 grams carrots daily.  
 Skinned and eviscerated carcass—847 grams.

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TABLE XII.  
*Rabbit 74—Injection of creatinine.*

DAYS	BODY WEIGHT	URINE			CREATININE INJECTED	CREATININE RECOVERED IN URINE
		Volume	Creatinine	Creatine		
	<i>kgms.</i>	<i>cc.</i>	<i>mgms.</i>	<i>mgms.</i>	<i>mgms.</i>	<i>per cent</i>
1	1.95	235	88	0		
2	1.96	210	88	0		
3	1.97	175	88	0		
4	1.91	65	88	0		
5	1.92	165	88	0		
Average.....			88			
6	1.95	265	174	0	100	71
7	1.94	260	152	0	100	
8	1.96	320	152	0	100	
9	1.91	290	257	0	200	80
10	1.91	285	243	0	200	
11	1.90	295	240	0	200	
Average.....			203		150	77

Male rabbit; ate 350 grams carrots daily.

Skinned and eviscerated carcass—1084 grams.

## THE FATE OF PROTEIN DIGESTION PRODUCTS IN THE BODY.

### II. DETERMINATION OF AMINO NITROGEN IN THE TISSUES.

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The methods will first be described. In the latter part of the paper some particular points will be discussed in detail.

I. *Method for closest absolute results.* The sample of tissue, which may weigh from 5 to 30 grams, is immediately after excision weighed to within 0.01 gram, covered with boiling hot water, to which 1 cc. of 50 per cent acetic acid per liter is added, and heated on the water bath until the proteins are completely coagulated. In case the piece is a large one it is cut into several with a pair of scissors, in order that it may heat through more readily. Coagulation is complete in twenty or thirty minutes. The pieces are then lifted out of the water with forceps or crucible tongs, minced fine with a food macerator, and returned to the same water. The heating is continued for about ten minutes, with occasional stirring, and the supernatant liquid is then decanted through a filter of glass wool. The tissue pieces are covered with a fresh portion of boiling acidified water, using 5 or 10 cc. for each gram of tissue, and the extraction repeated for five or ten minutes, when the solution is decanted through the same filter. The use of 4 or 5 successive portions of hot water in this manner insures complete extraction of the amino-acids in the tissues. The extracts are transferred to a 1-liter, double-necked distilling flask, and concentrated under diminished pressure to about 20 cc. The distillation may be run as rapidly as is possible without loss of solution by foaming. The concentrated solution is transferred with a minimum amount of water to an Erlenmeyer flask, and mixed with 9 to 10 volumes of 95 per cent ethyl alcohol, or half that amount of absolute

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ethyl or methyl alcohol. The alcohol precipitates a small amount of protein which is not coagulable by heat. The solution is allowed to stand over night to complete precipitation. It is then filtered through a folded filter, washed with 80 per cent alcohol, and returned to the distilling flask. A few drops of phenolphthalein solution are added, and enough 25 per cent sodium hydrate solution, drop by drop, to render the solution alkaline. It is then concentrated under diminished pressure to drive off both ammonia and alcohol. If the receiving flask is provided with a guard flask, as illustrated in this *Journal*, x, p. 21, 1911, containing  $\frac{x}{10}$  acid, the ammonia can be determined. In the tissues of the dog it amounts to about 10 mg. per 100 grams of fresh tissue. When the volume of the concentrating solution has been reduced to 10 or 20 cc. the distillation is interrupted, and enough 50 per cent acetic acid is added to acidify the solution. About 50 cc. of water are also added, in order to insure that the last traces of alcohol shall be driven off, and the concentration is continued. In case the amount of tissue analyzed exceeds 10 grams, the distillation may be continued in the same 1 liter flask, but if the sample is smaller the solution should now be transferred to a flask of 300 to 500 cc. The extracts are concentrated to a few cc., and are then transferred, with several small portions of water, to a measuring flask. The size of the latter depends on the weight of tissues taken for analysis. When the amount is under 10 grams we use a 10 cc. flask to hold the final solution; when over 10 grams have been taken, a 25 cc. flask is used.

The solution is now ready for the amino determination. One can take a 10 cc. portion and use the larger amino apparatus,<sup>1</sup> but we have found it in general somewhat more convenient to take 2 cc. portions and employ the micro-apparatus.<sup>2</sup> The length of time which the reacting solution should be shaken in order to drive off all the amino-acid nitrogen depends somewhat on the temperature. When the latter is 15–20° the time should be five to four minutes; for 20–25° it is three minutes; for 25–30°, two and a half to two minutes. It is preferable that the solution should be shaken vigorously with a motor and the time kept down to these limits, for the sake not only of rapidity, but of accuracy. The

<sup>1</sup> This *Journal*, xii, p. 275, 1912.

<sup>2</sup> *Ibid.*, xvi, p. 121, 1913.

reason for this is, that, even after removal of the ammonia, the extracts contain small amounts of urea and other substances which belong to the class of slowly reacting amines, and are therefore not  $\alpha$ -amino-acids. The amount of nitrogen which these amines give off in the time required for amino-acids to react completely is small, but if the reaction were allowed to run for an indefinite length of time instead of being kept to a definite minimum the error might be both large and variable. The correction for the amount of nitrogen given off by these amines while the amino-acids are being decomposed is ascertained in the same manner as in blood analysis,<sup>3</sup> by continuing the reaction, after the gas from the  $\alpha$ -amino-acids has all been driven off, for a length of time equal to that utilized in decomposing the amino-acids (two to five minutes according to the temperature), and then measuring separately the gas evolved during this second reaction period. The correction found is fairly constant at about 6 per cent of the total amino nitrogen obtained.

II. *Simpler method for accurate comparable results.* As the correction for amines other than  $\alpha$ -amino-acids is small and fairly constant, it can be left out without decreasing appreciably the constancy of results, or affecting the determination of differences in amino-acid content. The effect of the ammonia present is also small and practically constant. Consequently when, as in most physiological work, differences rather than strictly absolute results are desired, one can simplify the above outlined method by leaving out the determination of the correction and the removal of the ammonia. The results agree as well as when the ammonia is removed and other amines are corrected for, but are about 10 per cent higher. One must, under any conditions, however, accurately control the time of the reaction in the amino apparatus. In our own experimental work we have always removed the ammonia. After determining the correction for amines other than amino-acids in experiments with about 20 dogs, however, without finding that it varied appreciably under any conditions or added to the significance of the results, we ceased to utilize it.

The *accuracy* of the determination is limited, not so much by sources of error in the method, as by the fact that one cannot obtain absolutely homogeneous samples of tissue. Duplicate amino

<sup>3</sup> Van Slyke and Meyer: *This Journal*, xii, p. 402, 1912.

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determinations on the same solution of tissue extract usually agree as closely as one can read the volume of nitrogen gas in the burette. The error in the final amino determination is practically negligible. We have also convinced ourselves that there is very little error connected with the processes of extracting the tissues and concentrating the extracts in the manner outlined above. If one analyzes tissue which has been dried and pulverized, so that different samples of material have the same composition, results with different portions do not vary more than 1 or at most 2 mg. of amino nitrogen per 100 grams of fresh tissue, the amino figure for the latter usually falling between 40 and 80 mg. per 100 grams. When, however, different portions of an organ, such as the lobes of the liver, are taken as duplicate samples or when symmetrically placed organs, such as the right and left gracilis muscles or the two kidneys, are used, one must expect variations up to 10 per cent of the amount of amino nitrogen determined.

The two concentrations under diminished pressure involved in the analysis cannot be replaced by concentrations on the water bath. During the latter the extracts darken, and part of the amino nitrogen disappears. The effect on results is especially marked with liver extracts. If the latter are concentrated to dryness and then taken up in water a black solution results, which may show less than half the amino nitrogen originally in the extract.

The chief problems of the analysis were the complete extraction of the amino-acids from the tissues, and the removal of the proteins from the extract, both operations being necessarily performed under conditions which result in neither loss of amino-acids nor their formation by hydrolysis of the protein present. Folin and Denis in their methods for tissue analyses met these conditions by extracting the tissues with cold methyl alcohol. This method was not suitable for our purposes, however, as alcohol extracts the lipoids, and they may settle out of the final water solution in such masses that they mechanically hinder an accurate amino determination. Extraction with hot water is much more rapid than with alcohol, and the extract contains a relatively small amount of lipoids. It contains an appreciable amount of protein, however. For its removal we found the above described alcohol treatment the most satisfactory general method, although it makes two vacuum concentrations necessary, when one would be suffi-

cient if the proteins could be satisfactorily thrown out of the water extract by one of the usual precipitants. We tried a number of these, and found that metaphosphoric acid in particular gave results with muscle extracts which were satisfactory and agreed with those by the alcohol method, but that in liver extracts, presumably because of the glycogen present, it failed to precipitate the protein satisfactorily.

The only apparent objection to the hot water extraction is, that it might increase the amino nitrogen by hydrolysis of some of the proteins present. That such hydrolysis does not occur appears from the following experiment.

Muscles fresh from the thigh of a dog were cut into pieces with scissors and dried over sulphuric acid at a pressure of 0.2 mm. The dried muscles were pulverized and thoroughly mixed. Samples of 3 grams each were weighed out, placed in flasks with 100 cc. each of hot water, and allowed to digest at 100° for periods of five minutes, thirty minutes, and one, two, and three hours respectively. At the end of the period of digestion the water was decanted through a glass wool filter, and the residue in the flask washed with three 75 cc.-portions of water at 100°, each portion being allowed to remain five minutes on the muscle shreds. In case the action of hot water on the tissues produces a sufficiently rapid hydrolysis to become a factor in disturbing the accuracy of results it should make itself apparent by increased amino nitrogen content in the samples which were digested longest. That this was not the case is shown in the table below. The water extracts were concentrated and precipitated with alcohol, and, after the alcohol had been driven off, the entire solution was used for determination of amino nitrogen in the larger apparatus.

TABLE I.

PERIOD OF DIGESTION WITH HOT WATER	CC. NITROGEN GAS AT 20°, 760 MM.
5 minutes.....	11.4
30 minutes.....	11.4
1 hour.....	11.5
2 hours.....	11.2
3 hours.....	11.2

The results also indicate the accuracy of the methods, from the initial extraction to the final determination, when the samples are taken from homogeneous material.

The results of the following experiment exemplify some points of interest.

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Fresh muscle of 123 grams' weight was extracted with hot water and the water extract brought to 100 cc. The 100 cc. were divided into five portions of 20 cc. each. The non-coagulable protein was left in No. 1. From the others it was removed by the means indicated in the table. The solutions were freed from ammonia and eventually all brought to 25 cc., of which 2 cc. portions were used for duplicate determinations of amino nitrogen. Ten cc. portions were, furthermore, mixed with 10 cc. each of concentrated hydrochloric acid and heated twenty-four hours to completely hydrolyze the proteins and intermediate products present.<sup>4</sup> The hydrochloric acid was driven off as thoroughly as possible by concentrating in vacuum, and the ammonia boiled off in vacuum with calcium hydrate. The residual solutions were diluted to their original volume of 10 cc., and 2 cc. portions taken for amino determinations.

The methyl and ethyl alcohols used were "absolute;" the zinc chloride solution contained 5 grams of the chloride dissolved in 100 cc. of 80 per cent ethyl alcohol.

TABLE II.

NO.	METHOD OF PRECIPITATING PROTEINS IN 20 CC. WATER EXTRACT	AMINO N PER 100 GRAMS MUSCLE		PEPTIDE BOUND N
		Free	After hydroly- sis of extract	
1	Not precipitated.....	89	224	135
2	100 cc. methyl alcohol.....	68	100	32
3	100 cc. methyl alcohol+2 cc. ZnCl <sub>2</sub> solution.....	55	89	33
4	100 cc. ethyl alcohol.....	65	98	33
5	100 cc. ethyl alcohol+2 cc. ZnCl <sub>2</sub> solution.....	58	85	27

Alcohol alone added to the water extracts precipitates chiefly proteins and higher intermediate products. The free amino nitrogen precipitated by methyl alcohol was  $89 - 68 = 21$  mg. The peptide bound nitrogen was  $135 - 32 = 103$  mg. The ratio (free  $\text{NH}_2$  : peptide bound  $\text{NH}_2$ ) in the precipitate was, therefore, approximately 1 : 5. The ratio in animal proteins is usually about 1 : 12 (the animal proteins containing about 5 per cent of their nitrogen as free amino nitrogen, about 60 per cent more being set free by hydrolysis). The ratio 1 : 5 shows that the alcohol precipitate consisted, in part at least, of intermediate products, but that it could have contained little or no free amino-acid nitrogen. Similar

<sup>4</sup> Conditions for Complete Hydrolysis of Proteins, *This Journal*, xii, p. 295, 1912.



results are found with ethyl alcohol as a precipitant. It removes proteins and intermediate products, but leaves the amino-acids.

The action of zinc chloride, which when added to the alcoholic mixture precipitates some substances that alcohol alone does not, is different. It apparently precipitates some free amino-acids, without much affecting the intermediate products. The decrease in free amino nitrogen caused by adding the action of zinc chloride to that of alcohol (compare No. 2 with No. 3 and No. 4 with No. 5) is greater than that of the peptide bound nitrogen. One can also obtain a precipitate with zinc chloride and alcohol in a slightly acid solution of the products of complete acid hydrolysis of casein, and the precipitate removes part of the amino nitrogen from the solution. It would seem, therefore, that zinc chloride in alcoholic solution precipitates some amino-acid or acids. We have not further investigated the point, as it is of minor interest, but because of the above results and others like them we have not utilized zinc

TABLE III.

SAMPLE	WEIGHT OF SAMPLE	CC. N HCl TO NEUTRALIZE AMMONIA	AMMONIA N PER 100 GM. TISSUE	CC. N <sub>2</sub> FROM 10 CC. OF FINAL 25 CC. SOLUTION (Correction for blank amines placed below each result and subtracted)	TEMPERATURE	PRESSURE	DURATION OF REACTION WITH HNO <sub>3</sub>	MGM. AMINO-ACID N PER 100 GM. TISSUE	
								Corrected for other amines	Uncorrected
			mg.		deg.	mm.	min.		
Left triceps muscle . . . . .	14.98	5.1	10	6.75	21	758	4		
				0.40			4		
				6.35				60	64
Right triceps muscle . . . . .	20.37	7.9	11	8.70	23	760	4		
				0.60			4		
				8.10				56	60
Liver, lobe 1 . . . . .	11.81	3.9	9	6.55	22	758	4		
				0.40			4		
				6.15				72	76
Liver, lobe 2 . . . . .	19.62	7.2	10	10.60	21	758	4		
				0.50			4		
				10.10				73	77

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chloride as a precipitant. It would not be likely to affect the significance of comparative results, however, like those of Folin and Denis, because the proportion of amino nitrogen precipitated is quite constant.

The results in table III, taken from data in connection with Dog 17, serve as examples of the order of magnitude of the figures obtained, the volumes of gas measured, etc. The determinations were performed according to the "absolute" method, the ammonia being removed, and the correction for the amines, other than  $\alpha$ -amino-acids, being determined.

The following table, from analyses of another dog, gives some typical figures.

TABLE IV.

TISSUE	FREE AMINO N IN WATER EXTRACT, BEFORE REMOVAL OF PROTEINS NOT COAGULATED BY HEAT	AMINO N IN EXTRACT AFTER PRECIPITATION OF PROTEINS WITH ALCOHOL		
		Free $\text{NH}_2$	$\text{NH}_2$ after complete hydrolysis with $\text{HCl}$	Peptide bound N ( $\text{NH}_2$ freed by hydrolysis)
Gracilis muscle.....	50	38	61	23
Heart.....	50	37	61	24
Liver.....	75	61	89	28
Spleen.....	64	53	68	15
Kidney.....	73	65	77	12
Stomach.....	41	32	53	21
Duodenum.....	66	50	60	10

The results in the second and third columns show that the amino nitrogen found in the extract after treatment with alcohol (second column) comes chiefly from free amino-acids. In the case of even the simplest peptides, the dipeptides, the free amino nitrogen is doubled by hydrolysis, and in the primary albumoses it is increased about eight times. Here the increase is only 20 to 50 per cent. A mixture of amino-acids and albumoses such that 93 per cent of the free amino nitrogen belongs to the amino-acids and only 7 per cent to the albumoses, would give an increase of 50 per cent in the amino nitrogen on hydrolysis of the albumoses. Such a relation apparently exists in the tissue extracts as prepared for analysis; for even after the alcohol treatment they show the presence of traces of protein or higher intermediate products when

tested for biuret with the precautions given by van Norman.<sup>5</sup> The relations between the free amino nitrogen and the peptide bound nitrogen given in the above table are typical. A much larger proportion of peptide bound nitrogen is never found. One can, therefore, depend upon figures for amino nitrogen in the tissues obtained by the above method as representing with a fairly close degree of approximation the simple amino-acids.

#### SUMMARY.

The amino-acids are extracted from the tissues with hot water. Uncoagulated proteins in the extract are precipitated by alcohol. Alcohol and the slight amount of ammonia present in the extract are removed by concentration in vacuum, and the amino nitrogen in the residue is determined by the nitrous acid method. The rapidity with which the amino nitrogen reacts with nitrous acid, and the relatively small increase which it shows as the result of hydrolysis of the extract with hydrochloric acid, indicate that the amino nitrogen determined by the method outlined represents approximately the *free  $\alpha$ -amino-acids*. Only a few per cent of the amino nitrogen appears due to proteins or their intermediate products, and to amines not of protein origin. The correction for the latter can, when desirable, be readily determined.

<sup>5</sup> *Biochem. Journ.*, iv, p. 127, 1909.



5

## THE FATE OF PROTEIN DIGESTION PRODUCTS IN THE BODY.

### III. THE ABSORPTION OF AMINO-ACIDS FROM THE BLOOD BY THE TISSUES.<sup>1</sup>

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In our preliminary communication<sup>2</sup> we mentioned only the most recent articles on the fate of the products of protein digestion. In order that our work may appear in its proper relation to that which has previously been done in the field, it appears desirable to review the latter more fully before proceeding with the report of our own results.

The contributions on the subject appear to be most readily grouped around the different explanations of the fate of ingested protein which have served as working hypotheses. These hypotheses may be formulated as follows:

1. The ingested proteins are absorbed and incorporated into the body without undergoing any marked chemical change.
2. The food proteins are first hydrolyzed in the alimentary tract; the products of digestive hydrolysis are then absorbed into the blood and carried to the tissues.
3. The products are deaminized in the wall of the intestine before entering the circulation.
4. The products are synthesized into serum protein before entering the circulation. The serum proteins thus formed serve as nourishment for the tissues in general.

We will take up these hypotheses in their order, reviewing the work which appears most important in supporting or antagonizing each.

<sup>1</sup> The results in this paper were reported in abstract at the meeting of the Soc. Exper. Biol. and Med., Dec. 18, 1912. *Proceedings*, x, p. 38.

<sup>2</sup> This *Journal*, xii, p. 399, 1912.

1. The simplest, and apparently the earliest theory concerning the manner in which the proteins of the food reach the tissues and are incorporated into them, is that the proteins are absorbed, with little or no chemical change, directly into the circulation, from which they are taken by the tissues and incorporated into their substance. In support of this view the fact was demonstrated that unchanged proteins could be made to pass directly from the alimentary canal into the blood.<sup>3</sup> Egg albumin can even be absorbed in such amounts that it appears in the urine.

More thorough investigation has shown decisively, however, that the absorption of unchanged proteins is an abnormal process. From a knowledge of the proteolytic powers of the digestive tract attained by the famous experiments of Spallanzani and Beaumont with the gastric juice, the discovery of trypsin by Kühne,<sup>4</sup> and the work of many later investigators, including Cohnheim,<sup>5</sup> the discoverer of erepsin, one is justified in stating that the more deeply the processes in the alimentary tract have been studied the more thorough has the breakdown of proteins in normal digestion been found. Cohnheim's work on this point is particularly important. He fed meat to a dog with a duodenal fistula which permitted isolation of the products of digestion after they had passed the stomach and part of the intestine. The partially digested products obtained from the fistula were treated with erepsin for twenty-four hours, the action of this enzyme thus following, as in normal digestion, that of the gastric and pancreatic juices. The meat proteins were hydrolyzed so completely that all the arginine was free<sup>6</sup> and the nitrogen precipitable by phosphotungstic acid could not be further decreased by boiling with sulphuric acid.<sup>7</sup> No evidence of the presence of peptides or intermediate products could be found. It appears probable, therefore, that normal digestion proceeds in the intestinal lumen and wall until most, if not all, of the proteolytic products are reduced to the stage of free amino-acids. Abderhalden and his co-workers have isolated nearly all of the known

<sup>3</sup> For references see article by Cohnheim: *Zeitschr. f. physiol. Chem.*, xxxv, p. 397.

<sup>4</sup> *Virchow's Archiv*, xxxix, p. 155.

<sup>5</sup> *Zeitschr. f. physiol. Chem.*, xxxiii, p. 451.

<sup>6</sup> *Ibid.*, li, p. 415, 1907.

<sup>7</sup> *Ibid.*, xlix, p. 64.

amino-acids from intestinal contents, and have furthermore shown that protein which has been digested completely into amino-acids is as efficient as intact protein in maintaining the nitrogenous equilibrium and even the growth of dogs.<sup>8</sup> Abderhalden has also found positive evidence that intact protein is not normally absorbed into the circulation.<sup>9</sup> Injection of protein into the circulation results in the development of a proteolytic enzyme in the blood capable of hydrolyzing the injected protein. The fact that the normal blood is free from such enzymes shows that it does not absorb undigested proteins. Furthermore, the fact that it is difficult or impossible to develop an anaphylactic state by protein feeding is proof against absorption of proteins as such, even in small amounts. Also, attempts to find evidence of food proteins in the blood by the precipitin reaction have given negative results.<sup>10</sup>

2. The absorption of intact proteins being an untenable hypothesis, the simplest alternative explanation is that the "peptone," or the mixture of digestive products, is absorbed directly into the blood and conveyed to the tissues.<sup>11</sup> That this mode is not impossible is indicated by recent work of Buglia.<sup>12</sup> He found that completely digested flesh in amounts equivalent to a day's protein requirement could be injected intravenously into dogs without injurious effect if several hours were taken for the injection, so that the rate of entrance of the products was similar to the rate of absorption in normal digestion. The injected products were mostly metabolized and excreted as urea.

That during the actual protein digestion, however, the final hydrolytic products enter directly into the circulation without undergoing chemical change while passing the intestinal wall, remained uncertain because of the many failures to demonstrate these products in the normal blood. For several decades it has been investigated for the presence of peptone with negative results.<sup>13</sup> When

<sup>8</sup> *Synthese der Zellbausteine.*

<sup>9</sup> *Schutzfermente.*

<sup>10</sup> Debré and Porak: *Journ. phys. et path. gén.*, xiv, p. 1019.

<sup>11</sup> A clear presentation of this view was given in 1905 by Folin: A Theory of Protein Metabolism, *Amer. Journ. of Physiol.*, xiii, p. 117. A thorough discussion of work up to 1912 on this subject is given by Cathcart: *Physiology of Protein Metabolism*, chapter on protein regeneration.

<sup>12</sup> *Zeitschr. f. Biol.*, lviii, p. 162, 1912.

<sup>13</sup> Abderhalden and Oppenheimer: *Zeitschr. f. physiol. Chem.*, xlii, p. 155, 1904; Howell: *Amer. Journ. of Physiol.*, xvii, p. 273, 1906.

the importance of amino-acids as the end products of digestion became appreciated these also were sought, but the most careful work failed to result in the isolation of a single amino-acid from normal blood, even during the height of digestion.<sup>14</sup>

Abderhalden, Gigon, and London were able, it is true, after injecting alanine into the stomach of a dog, to isolate that amino-acid from the blood and urine as the naphthylsulpho compound.<sup>15</sup> Results likewise indicating the ability of protein digestion products to pass from the intestine into the circulation were obtained by Cathcart and Leathes.<sup>16</sup> They found an increase in the "residual nitrogen" (nitrogen left after subtraction of the urea and removal of the protein precipitable by heat and by tannic acid) following the absorption of peptone injected into a loop of the intestine of a dog. An increase in the non-protein nitrogen of the liver was also noted. Results on the blood similar to those of Cathcart and Leathes have recently been obtained with a refined technique by Folin and Denis<sup>17</sup> after injection of amino-acids into the intestines of cats. These authors have also determined an increase in the non-protein nitrogen of the muscles following the absorption of amino-acids from the intestine. These results, however, can hardly claim the same degree of finality as those of Folin and Denis on the question of deamination and urea formation to be shortly discussed. The interpretation of changes in non-protein nitrogen as changes in amino-acid nitrogen is arbitrary, as there was no evidence concerning the chemical nature of the nitrogen in which the changes were noted, aside from the proof that it was not in the form of urea or ammonia.

The fact that amino-acids *can* enter the blood from the alimentary canal when the latter has been flooded with them, although clearly demonstrated by Abderhalden, Gigon, and London, did not carry with it the proof that absorption of unchanged amino-acids into the circulation occurs during their gradual liberation in digestion, nor was it regarded as such proof by these authors. Egg albumin can also be made to enter the blood and even appear in the urine when the alimentary canal is flooded with it under properly

<sup>14</sup> Abderhalden: *Synthese der Zellbausteine*.

<sup>15</sup> *Zeitschr. f. physiol. Chem.*, liii, p. 113, 1907.

<sup>16</sup> *Journ. of Physiol.*, xxxiii, p. 463, 1906.

<sup>17</sup> *This Journal*, xi, p. 87, 1912.



chosen conditions. Proof of the presence of amino-acids in the blood under normal conditions none of the investigators in the field was able to accomplish, despite the application of all the methods known to modern chemistry for the isolation of these substances. Investigation of the residual nitrogen of the blood indicated the possibility of the absorption of amino-acids or peptides. The failure to ascertain the chemical nature of the fraction of residual nitrogen to which the changes noted were due, however, made the results inconclusive, as those based on residual nitrogen determinations noted in the preceding paragraph would also have been, had they not been confirmed by the more definite findings of Abderhalden, Gigon, and London. Hohlweg and Meyer<sup>18</sup> found in the serum of fasting dogs an average residual nitrogen of 7 mg. per 100 cc. In digesting animals the average figure was 13 mg. As the amounts were so small, however, the individual fluctuations considerable, and in particular as there was no evidence of the chemical nature of the residual nitrogen, the results could not be regarded as decisive. Howell attacked the problem<sup>19</sup> with the aid of naphthylsulfochloride, which had been introduced by Fischer and Bergell as a precipitant for amino-acids. He obtained a precipitate in the dialysate of the serum, and the bulk precipitated was observed to be larger in the serum of fed dogs than in that of fasting animals. The precipitate was an oil, however, which could neither be identified chemically nor measured quantitatively. For this reason, notwithstanding the interesting possibilities indicated by the results, it could not be stated with certainty what proportion, if any, of the precipitate was due to amino-acids. Cohnheim, working with the alimentary canal of the octopus under conditions which to some extent simulated the natural, succeeded in separating crystalline amino-acids from the blood.<sup>20</sup> The tract under normal conditions practically floats in the blood of the animal. Cohnheim removed the tract with the digestive glands attached, filled it with peptone solution, and floated it for twenty hours in blood through which oxygen was passed. The organs remained alive during the experiment. At the end of the latter the residual nitrogen of the blood, which is ordinarily almost nil, was found

<sup>18</sup> *Hofmeister's Beiträge*, xi, p. 381, 1908.

<sup>19</sup> *Amer. Journ. of Physiol.*, xvii, p. 273, 1906.

<sup>20</sup> *Zeitschr. f. physiol. Chem.*, xxxv, p. 396, 1902.

to be greatly increased. No peptone could be detected in it, but ammonia was determined, and leucine, tyrosine, and lysine picrate were crystallized from it, though not enough of any was obtained for analysis. Cohnheim was conservative about generalizing these results to apply to normal digestion in the higher animals, and his reserve appeared to be justified by later results which he obtained in repeating the above experiment with a vertebrate fish, *Crenilabrus pavo*. He obtained ammonia in the external blood, but no evidence of either mono- or diamino-acids, and concludes: "dass bereits beim Passieren der Darmwand die Eiweisspaltprodukte teilweise desamidiert werden und in Ammoniak und einen, zunächst unbekannten Rest zerfallen."<sup>21</sup>

3. In the view of the failure to obtain conclusive proof of the presence of amino-acids in the blood of the higher animals during digestion, the above results of Cohnheim suggested the possibility that the amino-acids are *deaminized while passing the intestinal wall*, the first stage of their catabolism occurring before they enter the circulation. This hypothesis derived support from earlier work of Nencki, Zaleski, Pavlov, Salaskin, and Horodynski, who found the ammonia content of the portal blood greater than that of the arterial during digestion.<sup>22</sup> None of these results proved, however, that the greater part of the amino-acids suffers decomposition during absorption, and the deaminization hypothesis has in all events been effectively retired by recent work of Folin and Denis.<sup>23</sup> Using delicate quantitative methods which they had developed for the determination of ammonia and urea, they found that neither of these products appeared in increased amounts in the blood during absorption of glycocoll or alanine from a loop of the small intestine of the cat. They also showed that the ammonia of the portal blood is due largely to the products of putrefaction in the intestine.

4. The remaining explanation of the means by which the products of protein digestion reach the tissues without appearing in the blood is the antithesis of the recently demolished deaminization hypothesis. It assumes that the products in passing the intestinal wall, instead of being decomposed, are *synthesized into protein again*, and

<sup>21</sup> *Zeitschr. f. physiol. Chem.*, lix, p. 239, 1909.

<sup>22</sup> For references, see Cohnheim: *ibid.*, lix, p. 246.

<sup>23</sup> This *Journal*, xi, p. 161, 1912.

that the result of the synthesis is one or more of the proteins of the serum. This explains at one stroke both the failure to find amino-acids in the blood and the origin of its proteins. The explanation was founded on less positive evidence than the deamination hypothesis, but its long life shows that it possessed the advantage of being difficult to disprove. The hypothesis was clearly formulated at least as early as 1870 by Funke:<sup>24</sup> "Da im Blute und im Chylus gar keine oder nur Spuren von Peptonen sich finden, so bleibt keine andere Annahme übrig, als die, dass die Peptone unmittelbar nach ihrer Aufsaugung, gleichviel ob dieselbe in's Blut oder Chylus oder beide stattfinde, in gewöhnliche Eiweisskörper, vielleicht in die gleiche Modification, das Serumalbumin, zurückverwandelt werden." Hoppe-Seyler localized the process of resynthesis even more definitely:<sup>25</sup> "Da nun in Magen und durch das Pankreassekret Acidalbumin und Pepton gebildet wird, so scheint auch das eine Funktion der Epithelzellen des Darmes zu sein, diese Körper in Serumalbumin und fibrinbildende Stoffe überzuführen." Evidence which might be interpreted in favor of the resynthesis theory was brought by Hofmeister,<sup>26</sup> who found that the stomach wall of a digesting dog contained peptone, which, however, disappeared rapidly when the stomach was kept for a half hour or more at 40°. Hofmeister pointed out that the disappearance could be due to either resynthesis or further digestion of the peptone. Glässner<sup>27</sup> decided the question in favor of resynthesis. He found that the disappearance of non-coagulable nitrogen was due entirely to the albumoses (fraction not precipitated by heat, but precipitated by saturation with zinc sulphate), the nitrogen in the filtrate from the albumoses, which contained the products of further digestion, remaining constant after removal of the stomachs from the animals. Embden and Knoop,<sup>28</sup> however, who repeated the experiment, with the difference that they used intestine instead of stomach, found that the decrease in albumoses was accompanied by an increase in their filtrate, and was therefore to be attributed

<sup>24</sup> *Lehrbuch der Physiologie*. Quoted by Popoff: *Zeitschr. f. Biol.*, xxv, p. 427, 1889. Popoff believed that the synthesis occurred in the lumen of the tract, before absorption.

<sup>25</sup> *Pflüger's Archiv*, vii, p. 399.

<sup>26</sup> *Zeitschr. f. physiol. Chem.*, vi, p. 69, 1882.

<sup>27</sup> *Hofmeister's Beiträge*, i, p. 329, 1902.

<sup>28</sup> *Ibid.*, iii, p. 120, 1903.

to further digestion of the albumoses rather than to their resynthesis into protein. This had been advanced by Cohnheim<sup>29</sup> as the probable explanation of Hofmeister's results, after Cohnheim had discovered the activity of the erepsin of the intestinal wall. Aside from the results of Glässner, which are opposite to those later obtained by Embden and Knoop, it appears that no positive evidence has ever been found for the hypothesis that the products of protein digestion are resynthesized in the walls of the alimentary canal into blood protein.

The real evidence, the failure to identify the products of protein digestion in the blood, has been purely negative. This evidence, we believe we are justified in stating, has, even if one cannot admit the conclusiveness of the significant work of Howell, been decisively eliminated by the results published by us a year ago.<sup>30</sup> Using the nitrous acid method for determination of amino groups under precautions which render it specific for  $\alpha$ -amino-acids, we found that the latter are always present in the blood of dogs, the amount of amino-acid nitrogen being 3 to 5 mg. per 100 cc. of blood in animals after twenty-four hours fasting. After a meal of meat the figure rose to 10–11 mg. in the same animals. The results not only dispelled the negative evidence on which, because of lack of sufficiently sensitive methods, the resynthesis hypothesis had been built, but afforded positive proof that the products of protein digestion enter directly into the circulation. The amount of amino-acid nitrogen present at any one time in the blood is small, because amino-acids which enter it leave it with great rapidity. We found that intravenously injected alanine disappeared from the circulation almost as fast as it entered. A similar disappearance of injected amino-acids had shortly before been already noted by Woelfel.<sup>31</sup>

Immediately after our paper one on the same subject by Abderhalden and Lampé appeared.<sup>32</sup> In their work the amino-acid nitrogen of the blood was detected by the ninhydrin color reaction, the intensity of the color developed affording comparative results.

<sup>29</sup> *Zeitschr. f. physiol. Chem.*, xxxiii, p. 451, 1901.

<sup>30</sup> *This Journal*, xii, p. 399, 1912.

<sup>31</sup> *Proc. Amer. Physiol. Soc.*, Abstracts, 1911, p. 38; published in *Amer. Journ. of Physiol.*, xxix, p. xxxviii, 1912.

<sup>32</sup> *Zeitschr. f. physiol. Chem.*, lxxxi, p. 473, 1912.

The results confirmed ours, but the authors still favor the resynthesis hypothesis, believing that the main portion of the digestion products is resynthesized into blood protein during absorption. The passage of amino-acids unchanged into the circulation during digestion they explain on the basis of the difference in composition between the proteins of the food and the blood respectively. The food proteins contain certain amino-acids in greater proportions than the blood proteins, and some of these amino-acids will necessarily be left over when the maximum amount of serum protein has been synthesized from the food. It is, according to Abderhalden and Lampé, only these superfluous amino-acids that pass unchanged into the circulation. The part of the absorbed products important for nutrition is that which enters the circulation as serum protein, and it is the serum protein, according to Abderhalden, that nourishes the tissues in general.<sup>33</sup> These take up the protein from the serum, hydrolyze it again into amino-acids, and from the latter reconstruct their own proteins. In regard to the ascertained facts (absorption of amino-acids directly into the circulation during digestion) there is no disagreement between Abderhalden and ourselves. The above hypothesis, however, notwithstanding the valuable work which it has stimulated, is not, it seems to us, the most probable explanation of the facts thus far at our disposal. It assumes a number of processes (synthesis of absorbed amino-acids in the intestinal wall to serum protein, utilization of serum protein as pabulum by the body cells) as yet quite undemonstrated by established facts. Moreover, the demonstrated mechanism, by which the amino-acids liberated during digestion are absorbed directly into the circulation and transferred to the tissues, is sufficient to handle these products as rapidly as they are formed; and we know at present of no ground for assuming additional and more complicated processes to provide the tissues with protein constituents. In brief, it has been found that an undetermined proportion, possibly all, of the amino-acids formed in digestion passes unchanged into the circulation; and it has not yet been shown that any of them, except such as may be altered by bacteria, are either conjugated or destroyed before entering the blood stream.

Recently a paper appeared by Rona<sup>34</sup> which, furthermore, offered

<sup>33</sup> *Synthese der Zellbausteine*.

<sup>34</sup> *Biochem. Zeitschr.* xlv, p. 307, 1912.

evidence that no large fraction of the amino-acids suffers chemical change while passing the intestinal wall. Surviving intestines of cats were suspended in Tyrode's salt solution and filled with solutions of amino-acids or of digested peptone, the amino nitrogen content of these solutions having been determined by the gasometric method. After several hours, during which the intestines maintained their vitality and motility, from one-half to one-third of the amino nitrogen had diffused through the intestinal walls into the Tyrode's solution. There was no decrease in the total amount of amino nitrogen present, such as would have occurred if the passage of the intestinal wall had been accompanied by a synthesis of protein. Although Rona himself did not claim that his results were conclusive, inasmuch as there was no circulation of blood through the intestines and conditions were therefore not entirely comparable to those in the living animal, absolutely negative results would hardly have been expected if the intestines normally possess the ability to synthesize protein at the rate necessary to keep pace with absorption.

From the above review it appears that the positive results of previous work on the problem before us can be condensed into the following statement: *Ingested proteins are hydrolyzed in the digestive tract setting free most, if not all, of their amino-acids. These are absorbed into the blood stream, from which they rapidly disappear as the blood circulates through the tissues.*

In the present paper we attempt to answer the question: *What becomes of the amino-acids when they vanish from the circulation?* Are they decomposed in the blood: are they at once synthesized into new protein; are they chemically incorporated into the complex molecules of the tissue proteins; or are they merely absorbed by the tissues in general, or by certain tissues in particular, without undergoing any immediate change?

#### EXPERIMENTAL.

*Experiment 1.* A male dog of 9 kg. weight, which had fasted four days, was etherized and kept with artificial respiration by the Meltzer and Auer insufflation method during the entire experiment. The bladder was washed out through a catheter, and a sample of 25 cc. of blood was drawn from the right femoral artery. The right gracilis muscle, a lobe of the liver, a short section of the

small intestine, and the right kidney were removed and coagulated for determination of amino nitrogen. The lobe of liver was isolated by means of a large clamp at the base before excision; the other samples were dissected and tied off, so that all were taken practically without loss of blood. One hundred and fifty cc. of a solution of the amino-acids obtained by hydrolysis of casein were then injected into the right femoral artery. The solution was made by boiling casein forty-eight hours with seven times its weight of 33 per cent sulphuric acid. The sulphuric acid was removed with excess barium hydrate, and the ammonia removed by concentrating the alkaline solution in vacuum. The barium was then removed with sulphuric acid, the reaction being so balanced that the filtrate from the barium sulphate gave a barely perceptible reaction for sulphate. This condition assured complete removal of the barium. The solution was concentrated in vacuum, and the slightly acid reaction was changed by adding sodium carbonate until the solution gave a barely perceptible alkaline reaction with litmus. The final solution contained 27.08 mg. of amino nitrogen per cc. It was used instead of the solution of a single amino-acid because it undoubtedly resembles more nearly the mixture of amino-acids absorbed from the intestine during digestion. The 150 cc. injected contained 4.06 grams of amino nitrogen.

The duration of the injection was thirty minutes. Half an hour after it had been finished another sample of 25 cc. of blood was drawn, the dog was killed by bleeding, and samples of the tissues again taken.

During the period following the injection 125 cc. of urine were voided through the catheter or expressed from the bladder at the end of the experiment.

The results of the analyses, which were made by the "absolute" method described in the preceding paper, are given in table I.

The blood analyses were made as described in our first paper.<sup>35</sup>

The urine excreted during the experiment contained 0.738 gram of nitrogen, of which 0.463 gram, or 11 per cent of the amount injected, was amino-acid nitrogen.<sup>36</sup> Calculating the blood as 5 per cent of the weight of the animal, the increase of 41.5 mg. in the

<sup>35</sup> This *Journal*, xii, p. 402, 1912.

<sup>36</sup> For method of determining amino nitrogen in urine, see Van Slyke: *ibid.*, xvi, p. 125, 1913.

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TABLE I.

TISSUE SAMPLE	MG. AMINO NITROGEN PER 100 GRAMS TISSUE	
	Before injection	Thirty minutes after injection
Liver 1.....	34	
Liver 2.....	29	
Liver 3.....		94
Liver 4.....		93
Gracilis muscle 1.....	43	
Gracilis muscle 2.....		67
Gracilis muscle 3.....		73
Kidney 1.....	45	
Kidney 2.....		106
Intestine 1.....	48	
Intestine 2.....		97
Pancreas.....		91
Spleen.....		81
Blood 1.....	3.9	
Blood 2.....		45.2
Blood 3.....		45.6

amino nitrogen indicates that 0.19 gram, or 5 per cent, of the injected amino nitrogen remained in the circulation at the end of the experiment. The intestinal juice measured, as nearly as could be estimated, 200 cc. It contained 45 mg. of amino nitrogen per 100 cc., or a total of 0.09 gram, 2 per cent of the amount injected. It is doubtful whether this small amount was due to excretion of part of the injected amino-acids into the intestine, or to traces of unabsorbed digestive products.

Summarizing the results of the experiment: *Of the amino nitrogen injected, approximately 5 per cent remained in the circulation a half an hour after the injection. Eleven per cent had been excreted in the urine. If the remaining 3.41 grams of amino nitrogen injected had been absorbed by the tissues evenly throughout the body, the average increase per 100 grams of tissue (taking the weight of tissues aside from the blood as 8.5 kg.) would have been 40 mg. The increases found were: in the muscles 27 mg.; liver, 60 mg.; kidney, 60 mg.; intestine, 50 mg. Although strictly accurate calculations are, of course, impossible, the results indicate, as closely as one can estimate from such figures, that all the amino-acids which disappeared from the circulation were absorbed, without suffering immediate chemical change, by the tissues.*



It will be noted that the amino nitrogen content of the muscles did not rise so high as that of the internal organs. The figures exemplify a fact that we have noted in all our experiments, viz., that the amount of amino-acid nitrogen that the muscles can hold is limited with relative sharpness. By injection of the amino-acids from proteins hydrolyzed by either acids or enzymes we have never been able to force the amino nitrogen figure of the striped muscle above 80 mg. per 100 grams. If the figure is above 70 at the time of injection, little or no amino nitrogen is taken up. In the liver we have noted as high as 160 mg., and the other internal organs seem to possess a more elastic ability to absorb amino-acids than do the muscles.

*Experiment 2.* The conditions were similar to those of the first experiment. The dog used for Experiment 2 weighed 7.4 kg., and the amount of amino nitrogen injected was 3.39 grams. In this case the second set of tissue samples was taken one hour after the injection had been finished.

TABLE II.

TISSUE SAMPLE	MG. AMINO NITROGEN PER 100 GRAMS TISSUE	
	Before injection	One hour after injection
Liver 1.....	48	
Liver 2.....	44	
Liver 3.....		127
Liver 4.....		124
Right gracilis 1.....	46	
Right gracilis 2.....	46	
Left gracilis 1.....		78
Left gracilis 2.....		76
Right kidney.....	52	
Left kidney.....		111
Blood 1.....	5.8	
Blood 2.....	5.9	
Blood 3.....		31.5

The amino nitrogen excreted in the urine during the diuresis following the injection was 0.552 gram, or 16.3 per cent of that injected.

Other experiments could be cited, but the above two appear to show with sufficient clearness the phenomena discussed in connection with the first experiment. The following serves as a control.

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*Experiment 3.* A female bull terrier of 17 kg. weight, which had fasted twenty-four hours, was given during an hour an intravenous injection of 250 cc. of physiological saline solution. Samples of tissues and blood were taken before and after the injection as in the previous experiment.

TABLE III.  
*Injection of salt solution.*

TISSUE SAMPLE	MG. AMINO NITROGEN PER 100 GRAMS TISSUE	
	Before injection	After injection
Right sartorius 1.....	59	
Right sartorius 2.....	67	
Left sartorius 1.....		61
Left sartorius 2.....		62
Liver 1.....	56	
Liver 2.....	54	
Liver 3.....		54
Liver 4.....		55
Liver 5.....		56
Kidney 1.....	43	
Kidney 2.....		43
Blood 1.....	4.9	
Blood 2.....		4.7

Diuresis followed the salt solution injection, 150 cc. of urine being excreted. The nitrogen excreted was 0.514 gram, of which, however, only 0.010 gram was amino-acid nitrogen. The concentration of the blood was controlled by determination of its total nitrogen content. It was practically the same (2.94 per cent) at the end of the experiment as at the beginning (2.92 per cent). The results in the above table show that the operation and injection of water solution cause no appreciable change in the amino nitrogen content of the blood or tissues.

Concerning the physiological effects of intravenous injection of amino-acid mixtures, we have noted from experiments, including others than those tabulated above, the following behavior. Mixed amino-acids, whether obtained by acid hydrolysis of casein or by artificial digestion of beef (with pepsin, trypsin, and erepsin, till 90 to 95 per cent of the maximum amount of  $\text{NH}_2$  that can be freed by acid hydrolysis has been liberated), are tolerated by dogs in

doses up to 0.15–0.20 gram of amino nitrogen per kilo body weight, one hour or more being taken to complete the injection. During the latter no serious fall in blood pressure occurs, respiration appears normal and the animals apparently uninjured, as found by Buglia after similar injections. When the amino nitrogen injected exceeds the above limits, it may cause trembling, weakened heart action, fall in blood pressure, and death within an hour or two after the injection. Diuresis is abundant in these cases, and we have noted the excretion of as much as 500 cc. of urine, containing 20 per cent of the injected amino nitrogen. Doses under 0.15 gram of amino nitrogen per kilo usually cause but relatively moderate diuresis and excretion of amino-acids.

We have stated that amino-acids entering the blood stream are merely absorbed without chemical alteration by the tissues. This is a very loose description of a phenomenon for which the complete explanation will be far from easy. That the absorbed amino-acids enter into the organic structure of the tissue proteins, however, seems to be positively excluded by the fact that they can be removed again by such mild means as extraction with, not only hot water, but with cold water or alcohol. Of purely physical explanations, osmosis can be definitely excluded, because the ultimate concentration in the tissues is several times higher than in the blood. That the amino-acids should pass, so to speak, up hill, from a medium where they are dilute to one where they are more concentrated, requires another explanation than mere osmosis. It is possible that, having diffused into the cells, the amino-acids are fixed in a loose molecular combination by the proteins, as water of crystallization is held by salts, or as, according to Pfeiffer's recent results, the amino-acids themselves combine with neutral salts such as sodium and calcium chlorides.<sup>37</sup> A second possible explanation which is not yet ruled out by the facts is that of purely physical adsorption, the amino-acids being attracted to the colloids of the tissues by forces of surface tension or molecular attraction, such as enable charcoal or cloth fibers to adsorb dyes. We shall, however, leave the solution of this phase of the problem to the future, and merely use the term "absorption" to designate the phenomenon.

<sup>37</sup> Pfeiffer: *Zeitschr. f. physiol. Chem.*, lxxxi, p. 329, 1913.

## SUMMARY.

The disappearance of intravenously injected amino-acids from the circulation is the result of neither their destruction, synthesis, nor chemical incorporation into the cell proteins. The acids are merely *absorbed from the blood by the tissues, without undergoing any immediate chemical change.* In the case of the muscles at least, a fairly definite saturation point exists, which sets the limit to the amount of amino-acids that can be absorbed. We have never been able to force the amino nitrogen figure of the striated muscles above 75–80 mg. per 100 grams. The capacity of the internal organs is more elastic; we have raised the amino figure of the liver to 125–150 mg.

The absorption of amino-acids from the circulation by the tissues, although extremely rapid, is never complete; the blood contains 3–8 mg. of amino-acid nitrogen per 100 cc. even after a fast of several days' duration. The amino-acids of the blood appear, therefore, to be *in equilibrium* with those of the tissues, a condition which accounts for all the observed phenomena, and would also account for any transfer of amino-acids which may occur from organ to organ, or from maternal organs to foetus.

The process by which the amino-acids are taken up and held by the tissues cannot be wholly osmotic, because the normal concentration of amino nitrogen in the tissues is five to ten times that in the blood; and even when the latter is suddenly loaded with injected amino-acids, they quickly gather in not equal, but greater, concentration in the tissues.<sup>38</sup> The most probable explanations of the process are, that it is either: (1) a mechanical adsorption, or (2) the formation of loose molecular compounds between the amino-acids and the tissue proteins, such as Pfeiffer has recently shown can be formed by the amino-acids themselves with inorganic salts. A discussion of this question would at present be premature.

<sup>38</sup> Further examples of this phenomenon are given in the experiments in the next paper.

## THE FATE OF PROTEIN DIGESTION PRODUCTS IN THE BODY.

### IV. THE LOCUS OF CHEMICAL TRANSFORMATION OF ABSORBED AMINO-ACIDS.<sup>1</sup>

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After tracing the amino-acids from the intestine into the blood,<sup>2</sup> and from the blood into the tissues,<sup>3</sup> the question next to offer itself concerns the duration of their stay in the various organs, and the nature of the changes which are responsible for their final disappearance. That the amino-acids do not long remain unaltered in the body follows from facts which are already known. Levene, working with Kober<sup>4</sup> and Meyer,<sup>5</sup> has found, for example, that alanine and arginine when fed to a dog are excreted almost completely in the form of urea in the next twenty-four hours. Also, when protein is added to the diet of an animal already in nitrogenous equilibrium, an increased excretion of urea follows, which during the next one or more days corresponds nearly to the amount of nitrogen in the added protein.

#### EXPERIMENTAL.

*Technique.* In order to obtain evidence on the point in question, experiments of the following nature were performed. Dogs were

<sup>1</sup> The results in this paper were reported in abstract at the meeting of the Society for experimental Biology and Medicine, Dec. 18, 1912. *Proceedings*, x, p. 38.

<sup>2</sup> This *Journal*, xii, p. 402, 1912.

<sup>3</sup> *Ibid.*, preceding article.

<sup>4</sup> *Amer. Journ. of Physiol.*, xxiii, p. 324.

<sup>5</sup> *Ibid.*, xxv, p. 214.

injected intravenously with non-toxic doses of glycocoll, or of the mixtures of amino-acids obtained by hydrolysis of casein or complete artificial digestion of meat, these mixtures corresponding more nearly to those normally absorbed from the intestine than could a solution of a single amino-acid. After the injection, which consumed from an hour to an hour and a half, a half-hour was allowed to elapse for the absorption of the injected amino-acids by the tissues to become approximately complete. Then samples of the tissues (liver, muscle, kidney, intestine) were ligated or clamped off and removed for analysis. Several hours later similar samples were taken in order to ascertain which organs had retained their stored amino-acids and which had metabolized them, or begun to do so, most rapidly. During the experiments the blood and urine were also controlled by analysis. The animals were kept under ether by the efficient and convenient insufflation method of Meltzer and Auer.

The animals were for some days before the experiments either fasted or fed on a protein-free diet, in order to remove the products of protein digestion from the alimentary canal, and also, if possible, to decrease the concentration of amino-acids in the tissues. The former object was, of course, easily obtained, but the latter was not. As will be shown in the next paper, a regular decrease in the amino-acids of the tissues cannot be caused by removal of protein from the diet.

The solutions injected were allowed to flow at a regular rate from a burette into either the femoral or jugular vein, and were warmed by passing through a glass coil in a bath at 40° just before they entered the vein. The animals were warmed with an electric pad. Samples of arterial blood were taken before the injection, a half hour afterwards, and at the end of the experiment, two to four hours later. The samples, of about 25 cc. volume, were mixed with a little powdered sodium citrate and 1 to 2 cc. of the complete blood taken with a pipette for Kjeldahl determination, to control the concentration. The remainder of each sample was freed from protein by precipitation with 10 parts of 95 per cent ethyl alcohol, as described in our preliminary paper.<sup>6</sup> The filtrate from the proteins was brought to 10 cc., of which 2-cc. portions were used for

<sup>6</sup> This *Journal*, xii, p. 402, 1912.

determination of amino-acid nitrogen, 1 cc. for urea determination by the recent method of Folin.<sup>7</sup>

Before the injection samples of either the gracilis or triceps muscles were taken and analyzed for amino nitrogen by the method described in our second paper.<sup>8</sup> The results serve to some extent to indicate the degree to which the tissues in general are saturated with amino-acids. The two samples after the injection, being used for comparison, were always the right and left gracilis or the right and left triceps. A gracilis cannot be accurately compared with a triceps (see following paper for examples) as the latter is usually somewhat higher in amino nitrogen. When kidneys were taken, either the entire organ was analyzed, or it was bisected longitudinally and the halves used for duplicates.

The amount of injected amino nitrogen, whether in the form of hydrolyzed casein, artificially digested meat, or pure amino-acids, was usually chosen at 0.15–0.20 gram per kilo body weight. Larger doses, although used in some experiments, are likely not to be tolerated by the animals (see Experiment 4, for example). Within a half-hour after the injection of the above amounts, the amino-acids have been almost completely absorbed from circulation by the tissues, the amino nitrogen content of the blood having fallen to 15–20 mg. per 100 cc. Samples of the tissues are then taken as described in a preceding paragraph.

Before the injections the animals were either catheterized and their bladders washed out, or cannulas were placed in the ureters. During the experiments the catheters were left in place in order to collect the urine that always begins to flow shortly after the injection is begun. The urine was collected in two periods, one extending from the beginning of the injection till the first tissue samples were taken after it; the other period being the remaining time of the experiment. The urines were analyzed for free amino-acid nitrogen,<sup>9</sup> urea,<sup>10</sup> ammonia, and total nitrogen, and the rotations were taken. For the latter, 2 cc. of concentrated hydrochloric acid were added to 10 cc. of urine; the mixture was cleared with

<sup>7</sup> This *Journal*, xi, p. 507, 1912.

<sup>8</sup> *Ibid.*, present number.

<sup>9</sup> Levene and Van Slyke: *ibid.*, xii, p. 301, 1912; Van Slyke: *ibid.*, preceding number.

<sup>10</sup> Folin: *ibid.*, xi, p. 507, 1912.

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charcoal, and the rotation taken in a 2 dm. tube with yellow light from a spectroscope. In urines of dogs that had fasted before the injections the rotation usually corresponded to that of a solution of the injected amino-acids containing amino nitrogen in the same concentration as the urine. When the animals had received a protein-free diet of fat and starch, however, the urines were much more strongly dextrorotatory, and contained reducing sugar.

In part of the experiments, after the first blood sample had been drawn from the carotid artery the latter was connected with a mercury manometer, and the blood pressure during the injection and succeeding hours recorded. Amino-acid mixtures containing sufficient nitrogen to keep the animals in equilibrium for twenty-four hours could usually be injected without causing a drop in blood pressure. Opening the abdomen and manipulating the viscera to remove samples caused a quick drop to, as a rule, about 60 mm. Subsequently the original blood pressure was partly, sometimes almost entirely, reestablished.

All duplicate analyses reported were made on separate portions of blood or tissue.

*Experiment 1.* The animal, a rather lean bitch in good condition, was fasted four days before the experiment, its weight falling from 11 kilos to 10.3. The animal was etherized, samples of the right gracilis muscle removed, and blood samples drawn from the right femoral artery. A catheter was inserted and the bladder washed out. A solution of hydrolyzed casein<sup>11</sup> containing 2.70

<sup>11</sup> Casein was hydrolyzed by forty-eight hours' boiling with 7 parts of 33 per cent sulphuric acid. The latter was removed with an excess of barium hydrate, and the ammonia driven off by concentration in vacuum. The barium was removed with sulphuric acid, just enough of the latter being used so that a slight test for sulphate could be detected in the solution. The latter was concentrated in vacuum, and made just perceptibly alkaline to litmus with sodium carbonate. The rotation of the solution was determined on the basis of the amino nitrogen content, in order that the results might be used for comparison with those of urine analyses. 1.000 cc. of the solution was mixed with 2 cc. of concentrated HCl, diluted to 10 cc., and cleared with charcoal. In a 2 dm. tube the rotation was +0.50°. The amino nitrogen content of the tenfold diluted solution was 0.00271 gram per cc. The rotation, calculated by the formula,

$$\alpha_{NH_2} = \frac{\text{observed rotation of 1 dm. layer of solution}}{\text{grams amino N per cc.}} \text{ is } \frac{0.25^\circ}{0.00271} = +92^\circ.$$



grams of amino nitrogen in 250 cc. was injected into the femoral vein during one hour and ten minutes. Shortly after the injection was begun urine began to drop from the catheter; the amount passed during the injection and the succeeding half hour was 260 cc.; during the next two hours only 25 cc. additional were excreted.

An hour after the injection had been finished samples of the right triceps muscle and a lobe of the liver were removed. Two and a half hours later the animal was bled to death; samples of the left triceps and of other lobes of the liver being taken. The results are given in Table I.

The blood pressure of this animal was not followed, but it seemed to tolerate without difficulty the unusually large dose of amino-acids, 0.26 gram of amino nitrogen per kilo. The analyses were made by the "absolute method" described in the second paper of this series.

TABLE I.

Weight of dog, 10.3 kilos. Amino nitrogen injected, 2.70 grams. Amino nitrogen excreted, 0.55 gram.

TISSUE SAMPLE	TIME OF EXCISION	AMINO NITROGEN PER 100 GRAMS TISSUE
		<i>mgm.</i>
Muscle. Right gracilis 1.....	Before injection.	45
Muscle. Right gracilis 2.....	Before injection.	47
Muscle. Right triceps 1.....	1 hour after injection.	68
Muscle. Right triceps 2.....	1 hour after injection.	63
Muscle. Left triceps 1.....	3 hours after injection.	67
Muscle. Left triceps 2.....	3 hours after injection.	65
Liver 1.....	1 hour after injection.	79
Liver 2.....	1 hour after injection.	85
Liver 3.....	3 hours after injection.	34
Liver 4.....	3 hours after injection.	39

The peptide-bound amino nitrogen in all the tissue samples was also determined, but it showed no significant changes.

The urine in this case shows, unlike that of most fasted animals, a much higher rotation than would be calculated from the content of amino-acids. The reducing power of this urine was not determined.

At the end of the experiment 5 cc. of bile were expressed from the gall bladder. Neither the bladder nor the intestine indicated that

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an unusual excretion of bile had occurred. The amino nitrogen content, determined without removal of the mucin, was only 0.12 mg. per cubic centimeter. The amino-acids which disappeared from the liver could not, to a significant extent, have been excreted in the bile.

TABLE II.  
*Blood analyses. Experiment 1.*

NO.	TIME	AMINO N PER 100 CC.	UREA N PER 100 CC.
		mgm.	mgm.
1	Before injection.....	8.2	11
2	Before injection.....	7.8	12
3	1 hour after injection.....	18.3	22
4	1 hour after injection.....	18.6	21
5	3 hours after injection.....	12.4	23
6	3 hours after injection.....	11.9	23

TABLE III.  
*Urine analyses. Experiment 1.*

	URINE FROM BEGIN- NING OF INJECTION TILL 1 HOUR AFTER INJECTION	URINE DURING NEXT 2 HOURS
Volume.....	260 cc.	25 cc.
Total nitrogen.....	0.854 gram	0.045 gram
Amino nitrogen.....	0.530 gram	0.018 gram
Observed rotation in 2 dm. tube of urine diluted with 0.25 volume con- centrated HCl.....	+0.50°	
* $\alpha_{NH_2}$ .....	+153.00°	

\* See footnote 11, page 216.

We interpret the results as follows. As the result of the injection the amino figure of the muscles rose to 66 and remained near the latter point for the following three hours, no noticeable amounts of the absorbed amino-acids being destroyed or synthesized into protein in the muscles, or removed from them during this period. In the liver the case is altogether different. As the result of absorption of injected amino-acids the concentration in the liver rose, as usual (see preceding paper), even higher than in the muscles. It fell again with almost startling rapidity, however, dropping between the second and fourth hours after the injection from 79-85

mg. to 34-39 mg. per 100 grams of fresh tissue. The latter figure is undoubtedly about as low as before the injection, for we have never found the amino figure in the livers of normal dogs, fasted or fed, below 30 mg. Presumably the fall had already begun before the first samples were taken, one hour after the end of the injection, two hours after it had been begun. For the nature of the change involving the disappearance of injected amino-acids from the liver the following explanations might be proposed.

1. The amino-acids were excreted. This explanation is entirely inadequate. The bile contained very little amino nitrogen, including that of its protein; and in the urine only 18 mg. of amino nitrogen were excreted during a period when 250-300 mg. disappeared from the liver.

2. The amino-acids were transferred to other tissues. This seems most improbable. None of the other large organs shows a greater avidity for amino-acids, to judge from the amounts absorbed (see paper preceding this), yet three or four hours after the injection all usually contain much more than the liver (compare following experiments). That the absorbed amino-acids should have been removed from the liver and concentrated in the other organs, after all had in free competition taken up their shares from the blood, is improbable.

3. The absorbed amino-acids are synthesized into body protein in the liver. Concerning this possibility we have at present no evidence on which we can decide in one way or another. It is possible that at least a part of the amino-acid mixture is immediately resynthesized into protein. There is, however, at present no positive evidence that this is the case. If a rapid synthesis were occurring in the liver one might expect to find an increase in the intermediate products indicated by the peptide bound nitrogen in the extract. We found no such increase, this nitrogen being about 20 mg. one hour, and the same three hours after the injection. Furthermore, the fall in the amino nitrogen of the liver following the injection of glycocoll is similar to that observed after injection of hydrolyzed protein (see Experiment 5). That glycocoll by itself should be turned into body protein appears impossible.

4. The amino-acids are deaminized with formation of urea or ammonia. If ammonia is formed it presumably undergoes further

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transformation at once; for during the disappearance of amino-acids from the liver we have been able to find no increase in the ammonia content of that organ (see next experiment), nor is the ammonia excretion in the urine marked. That at least part of the amino-acid nitrogen is transformed into urea is, however, definitely indicated by the increase in urea nitrogen of the blood following the injection of amino-acids.

The volume of the blood in the dog is, at a rough approximation, equal to that of the liver. In case the amino-acid nitrogen which disappears from the latter were transferred entirely and exclusively to the blood in the form of urea, we should expect the urea concentration of the blood to show a corresponding increase (only a small amount of urea escapes by way of the urine during the last period of the experiment). As a matter of fact, only a fraction of the amino nitrogen which disappears from the liver reappears as urea in the blood. It is possible, however, that the urea in the blood is, like the amino-acids, in equilibrium with that of the tissues, in which case urea entering the blood from the liver would, unless immediately excreted, be partially taken up by the other tissues. In a number of experiments we have analyzed the tissues for urea in order to test this point, but are not sufficiently satisfied with the reliability of the methods to report the results at present.

*Experiment 2.* The conditions were similar to those in Experiment 1, the chief difference being that the dose of amino-acids was smaller, so that the results were somewhat less pronounced. The animal, having fasted six days, weighed 12.8 kilos. He was etherized, and, as before, samples of blood and muscle were taken. 125 cc. of hydrolyzed casein, containing 1.90 grams of amino nitrogen (0.149 gram per kilo) were injected into the right femoral vein, one hour being taken for the injection. Samples of blood and tissues were taken thirty minutes after the injection was finished, and again three hours later.

The total amino nitrogen excreted was 0.12 gram, 6 per cent of the amount injected. The rotation of the chief fraction of the urine calculated on the basis of the amino nitrogen agrees closely with that of the injected solution, indicating that the different amino-acids were probably excreted in nearly the same proportions in which they were injected.

The results of this experiment demonstrate the same facts as the one preceding. In addition, the analyses of samples of kidney,

TABLE IV.

*Tissue analyses. Experiment 2.*

Weight of dog, 12.8 kilos. Amino nitrogen injected (hydrolyzed casein), 1.90 grams. Amino nitrogen excreted, 0.12 gram.

TISSUE SAMPLE	TIME OF EXCISION	AMINO NITROGEN PER 100 GRAMS	AMMONIA NITROGEN PER 100 GRAMS
		mgm.	mgm.
Right gracilis 1.....	Before injection.	49	12
Right gracilis 2.....	Before injection.	49	13
Right triceps 1.....	0.5 hour after injection.	65	13
Right triceps 2.....	0.5 hour after injection.	68	13
Left triceps 1.....	3.5 hours after injection.	66	12
Left triceps 2.....	3.5 hours after injection.	67	14
Liver 1.....	0.5 hour after injection.	58	10
Liver 2.....	0.5 hour after injection.	57	11
Liver 3.....	3.5 hours after injection.	33	12
Liver 4.....	3.5 hours after injection.	33	10
Pancreas.....	3.5 hours after injection.	63	9
Kidney.....	3.5 hours after injection.	50	10
Duodenum.....	3.5 hours after injection.	64	12

TABLE V.

*Blood analyses. Experiment 2.*

NO.	TIME TAKEN	AMINO N PER 100 CC.	UREA N PER 100 CC.	TOTAL N PER 100 CC.
		mgm.	mgm.	grams
1	Before injection.....	4.2	8	3.25
2	Before injection.....	3.9	8	
3	0.5 hour after injection.....	19.8	11	2.91
4	0.5 hour after injection.....	19.6		
5	3.5 hours after injection.....	7.5	14	3.14
6	3.5 hours after injection.....	8.2	13	

TABLE VI.

*Urine analyses. Experiment 2.*

	URINE FROM BEGIN- NING OF INJECTION TILL ONE-HALF HOUR AFTER INJECTION	URINE DURING NEXT THREE HOURS
Volume.....	43 cc.	12 cc.
Total nitrogen.....	0.213	
Amino nitrogen.....	0.100	0.021
Urea + ammonia nitrogen.....	0.058	0.018
Observed rotation in 2 dm. tube of urine diluted with 0.2 volume con- centrated HCl.....	+0.41°	+0.59°
* $\alpha_{NH_2}$ .....	+88°	+111°

\* See footnote 11, page 216.

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pancreas, and intestinal wall taken three and one-half hours after the injection indicate that deaminization in these tissues is not so rapid as in the liver, for the amino nitrogen content in none of them has sunk so low as in the liver.

*Experiment 3.* This experiment was similar to the preceding except that instead of the mixture of amino-acids obtained by acid hydrolysis of casein, that obtained by digestion of meat with pepsin, trypsin, and erepsin was injected.<sup>12</sup> The animal was kept for eight days before the experiment on a protein-free diet of starch, lard, and salts. The animal weighed 13.3 kilos before the protein-free diet was begun, 11.6 kilos at the time of the experiment. The bladder was washed out through a catheter as usual. The blood samples were drawn from the carotid artery, and the injection made into the jugular vein. The 185 cc. of solution injected contained 2.06 grams of amino nitrogen, a dose of 0.18 gram per kilo.

The muscles of this animal were, to judge from their high amino nitrogen content before the injection, filled with amino-acids before the experiment to a degree unusually close to saturation (see summary of preceding paper). This is probably the reason why the comparatively moderate injection of amino-acids forced the amino content of the liver to such an unusual height. As the result of this height, the succeeding drop is exceptionally great.

There was apparently no change in the amino-acid content of the kidneys in the period between thirty minutes and four hours after the injection. There was also little excretion of urine (7 cc.) during this period, although 240 cc. had been excreted during the injection and the first half hour thereafter.

From comparison of the triceps muscle before injection with the gracilis after (Table VII), one might judge that the muscle had taken up none of the injected amino-acids, as the figures are about equal before and after. Reference to the table in the next paper

<sup>12</sup> Beef was ground in a machine and boiled. It was then digested a week with pepsin, two weeks with trypsin, which was added in fresh portions every few days, and a month with the mucous membrane of dogs' intestines. Toluene and chloroform together were used to insure antisepsis. The course of the digestion was followed by amino determinations. At the time it was stopped it was 90 per cent complete, taking the amino nitrogen freed by boiling twenty-four hours with 20 per cent HCl as indicating 100 per cent. The antiseptics were removed by concentration in vacuum, and the solution boiled, then preserved at 0°.

TABLE VII.

*Tissue analyses. Experiment 3.*

Weight of dog (eight days on protein-free diet), 11.6 kilos. Amino nitrogen (digested beef) injected, 2.06 grams. Amino nitrogen excreted, 0.318 gram.

TISSUE SAMPLE	TIME OF EXCISION	AMINO N PER 100 GRAMS
Muscle. Triceps 1.....	Before injection.	70
Muscle. Triceps 2.....	Before injection.	65
Muscle. Right gracilis 1...	0.5 hour after injection.	67
Muscle. Right gracilis 2...	0.5 hour after injection.	73
Muscle. Left gracilis 1....	4 hours after injection.	70
Muscle. Left gracilis 2....	4 hours after injection.	73
Liver 1.....	0.5 hour after injection.	156
Liver 2.....	0.5 hour after injection.	157
Liver 3.....	4 hours after injection.	69
Liver 4.....	4 hours after injection.	73
Right kidney.....	0.5 hour after injection.	88
Right kidney.....	4 hours after injection.	89

TABLE VIII.

*Blood analyses. Experiment 3.*

NO.	TIME	AMINO N PER 100 CC.	UREA N PER 100 CC.	TOTAL N PER 100 CC.
		mgm.	mgm.	grams
1	Before injection.....	4.7	5	3.33
2	0.5 hour after injection.....	13.7	10	3.28
3	4 hours after injection.....	11.0	14	3.21

TABLE IX.

*Urine analyses. Experiment 3.*

	URINE FROM BEGINNING OF INJECTION TILL 1 HOUR AFTER INJECTION	URINE DURING NEXT THREE AND ONE-HALF HOURS
Volume.....	240 cc.	7 cc.
Total nitrogen.....	0.826 gram	0.025 gram
Amino nitrogen.....	0.318 gram	
Urea nitrogen.....	0.107 gram	
Ammonia nitrogen.....	0.024 gram	

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shows, however, that the amino-acid nitrogen in the triceps muscles is normally 10–20 mg. higher than in the gracilis. The figures in Table VII indicate, therefore, that the amino nitrogen of the gracilis was probably raised at least 10 mg. per 100 gm. by the injection.

*Experiment 4.* This experiment shows the typical results of an overdose of injected amino-acids. The animal, a female in the early stage of pregnancy, was on a protein-free diet for nine days before the experiment. 250 cc. of a solution of hydrolyzed casein, containing 3.81 grams of amino nitrogen, were injected. The dose,

TABLE X.

### *Tissue analyses. Experiment 4.*

Weight of dog (nine days on protein-free diet), 17.4 kilos. Amino nitrogen (hydrolyzed casein) injected, 3.81 grams. Amino nitrogen excreted, 0.873 gram.

TISSUE SAMPLE	TIME OF EXCISION	AMINO NITROGEN PER 100 GRAMS
Muscle. Right gracilis . . . .	Before injection.	36
Muscle. Right triceps . . . .	0.5 hour after injection.	56
Muscle. Left triceps . . . . .	2½ hours after injection.	55
Liver 1 . . . . .	0.5 hour after injection.	53
Liver 2 . . . . .	0.5 hour after injection.	58
Liver 3 . . . . .	2½ hours after injection.	45
Liver 4 . . . . .	2½ hours after injection.	48
Right kidney 1 . . . . .	0.5 hour after injection.	60
Right kidney 2 . . . . .	0.5 hour after injection.	69
Left kidney 1 . . . . .	2½ hours after injection.	48
Left kidney 2 . . . . .	2½ hours after injection.	43
Duodenum . . . . .	2½ hours after injection.	53
Spleen . . . . .	2½ hours after injection.	76
Pancreas . . . . .	2½ hours after injection.	66

0.21 gram of amino nitrogen per kilo, was but slightly above the usual tolerated amount. This animal, however, reacted at once with a profuse diuresis, 450 cc. of urine being excreted during the ninety minutes of the injection and the following thirty minutes. 200 cc. of normal saline solution were injected to replace the volume of water lost by the animal. The diuresis continued, 155 cc. more of urine being voided during the succeeding two hours. The heart weakened before the injection was finished and the breathing became shallow and irregular. Two hours after the finish of the



TABLE XI.  
*Blood analyses. Experiment 4.*

NO.	TIME	AMINO N PER 100 cc.	UREA N PER 100 cc.	TOTAL N PER 100 cc.
		<i>mgm.</i>	<i>mgm.</i>	<i>grams</i>
1	Before injection .....	10	10	3.32
2	Before injection .....	9	11	
3	0.5 hour after injection .....	31	16	3.03
4	0.5 hour after injection .....	30	16	
5	2½ hours after injection .....	20	14	3.40
6	2½ hours after injection .....	20	14	

TABLE XII.  
*Urine analyses. Experiment 4.*

	URINE FROM BEGIN- NING OF INJECTION TILL 0.5 HOUR AFTER INJECTION	URINE FROM 0.5 HOUR AFTER INJECTION TILL EXITUS
Volume.....	450 cc.	155 cc.
Total nitrogen.....	1.163 grams	0.362 gram
Amino nitrogen.....	0.658 gram	0.215 gram
Urea nitrogen.....	0.191 gram	0.033 gram
Ammonia nitrogen.....	0.070 gram	0.019 gram
Observed rotation (2 dm. tube) of urine diluted with 0.2 volume con- centrated HCl.....	+1.02°	+0.92°
Glucose (reduction).....	0.87 per cent	0.77 per cent
Rotation due to amino-acids, calcu- lated from $\alpha_{NH_2} = +92^{\circ}$ *.....	+0.26°	+0.24°
Rotation not due to amino-acids....	+0.76	+0.68
Glucose calculated from rotation not due to amino-acids.....	0.87 per cent	0.79 per cent

\* See footnote 11, page 216.

injection a convulsion occurred, and forty-five minutes later the heart stopped.

The animal exhibited an abnormal behavior in a number of ways. Despite the unusual proportion (23 per cent) of the injected amino nitrogen excreted in the urine, the amino content of the blood did not return so rapidly nor so nearly to normal as in the preceding experiments. The urea of the blood, instead of increasing somewhat during the last period, fell 2 mg.

As usual with dogs fed on fat and carbohydrate before the in-

jection, this was followed by a marked glucosuria, shown by both rotation and reducing power of the urine. Urine excreted shortly before the injection contained no sugar.

The nitrogen of the urine belonged chiefly to the amino-acids. In the hydrolyzed casein, freed of ammonia, which was injected, only 80 per cent of the total nitrogen is in the form of  $\text{NH}_2$ , the other 20 per cent being due to the proline, arginine and other amino-acids containing non-amino nitrogen. Therefore, the free amino nitrogen must be increased by one-fourth in order to calculate approximately the actual amount of amino-acid nitrogen present. The 0.658 gram of  $\text{NH}_2$  nitrogen excreted in the first period indicates that actually 0.82 of the 1.16 grams of nitrogen excreted was in amino-acids.

Both absorption of amino-acids by the liver (to judge from its analysis thirty minutes after the injection) and the subsequent decrease were small. In the kidneys, on the other hand, a marked fall occurred in the amino nitrogen content during the last period ( $\frac{1}{2}$  to  $2\frac{1}{4}$  hours after the injection). The most probable explanation of this seems to be that the amino-acids were washed out of the remaining kidney (one having been removed thirty minutes after the injection) by the active diuresis. A similar fall has been observed in two other experiments (unpublished), in each of which the diuresis continued into the last period of the experiment. Usually diuresis ceases shortly after the injection, and the amino content of the kidney does not fall markedly during the last period. We have other experiments under way to determine whether the explanation suggested is correct or not.

*Experiment 5.* This experiment was similar to the others, except that glyocoll instead of the mixture of amino-acids obtained by hydrolysis of a protein, was injected. The results were similar to those obtained with moderate doses of the mixtures. A rapid disappearance of absorbed amino-acids occurred from the liver, but not from the muscles or kidney. The spleen and pancreas were found loaded with amino nitrogen at the end of the experiment.

The injected solution contained 9.3 grams of glyocoll in 125 cc. of water, the dose of amino nitrogen per kilo (weight of dog, 10.9 kg.) being 0.16 gram. The injection was made into the jugular vein, one and one-half hours being taken to complete it. The

blood pressure remained at 145–150 mm. during the injection. When the liver samples were taken after the injection the pressure dropped to 60 mm. It rose again to 105 mm. during the next hour and a half, and remained at 95–105 during the rest of the experiment.

TABLE XIII.

*Tissue analyses. Experiment 5.*

Weight of dog (two days fast), 10.9 kilos. Amino nitrogen injected (glycocoll), 1.74 grams. Amino nitrogen excreted, 0.06 gram.

TISSUE SAMPLE	TIME OF EXCISION	AMINO NITROGEN PER 100 GRAMS
Right gracilis muscle 1 . . . . .	30 minutes after injection.	62
Right gracilis muscle 2 . . . . .	30 minutes after injection.	68
Left gracilis muscle 1 . . . . .	3 hours after injection.	67
Left gracilis muscle 2 . . . . .	3 hours after injection.	61
Liver 1 . . . . .	30 minutes after injection.	61
Liver 2 . . . . .	30 minutes after injection.	57
Liver 3 . . . . .	3 hours after injection.	43
Liver 4 . . . . .	3 hours after injection.	43
Kidney 1 . . . . .	30 minutes after injection.	68
Kidney 2 . . . . .	3 hours after injection.	65
Duodenum . . . . .	3 hours after injection.	66
Spleen . . . . .	3 hours after injection.	129
Pancreas . . . . .	3 hours after injection.	169

TABLE XIV.

*Blood analyses. Experiment 5.*

TIME	AMINO N PER 100 CC.	UREA N PER 100 CC.	TOTAL N PER 100 CC.
	mgm.	mgm.	grams
Before injection . . . . .	5.6	14	3.99
30 minutes after injection . . . . .	22.5	16	3.80
3 hours after injection . . . . .	13.4	27	3.89

The urine excreted measured only 25 cc. in all, and contained 0.203 gram nitrogen, of which but 0.061 gram was amino-acid nitrogen, this amount being 3.5 per cent of that injected. The tendency to excrete injected amino nitrogen appears to be somewhat less when the single amino-acid, glycocoll, is injected than when an equal dose in the form of the mixture of amino-acids obtained by hydrolysis of a protein enters the circulation.

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*Experiment 6. Control, saline injection.* This experiment was performed in order to ascertain whether the operative treatment and injection of liquid could have produced any changes in the amino nitrogen of the liver or kidney. A dog of 11 kg. received in one hour 200 cc. of normal saline, which was injected into the jugular vein. Thirty minutes after the injection was finished a lobe of the liver and one of the kidneys were removed for analysis. Three hours later similar samples were taken. The results in the table below show no changes in the amino figures during this period.

TABLE XV.

*Tissue analyses. Experiment 6.*

Weight of dog (fasted twenty-four hours), 11 kilos. Injection of 200 cc. of 0.8 per cent NaCl solution, no amino nitrogen.

TISSUE SAMPLE	TIME OF EXCISION	AMINO NITROGEN PER 100 GRAMS
Liver 1.....	0.5 hours after injection.	42
Liver 2.....	0.5 hour after injection.	47
Liver 3.....	3.5 hours after injection.	45
Liver 4.....	3.5 hours after injection.	41
Liver 5.....	3.5 hours after injection.	42
Right kidney.....	0.5 hour after injection.	48
Left kidney.....	3.5 hours after injection.	48

The amino acid nitrogen of the blood remained unchanged throughout the experiment at 5 mg. per 100 cc., and the urea nitrogen at 8 mg.

### CONCLUSION.

In the preceding paper we have shown that amino-acids injected into the circulation are absorbed by the tissues. In the present paper it is shown that the absorbed amino-acids (glycocoll, hydrolyzed casein, artificially digested flesh) disappear rapidly from the liver. The amino nitrogen content of this organ may be doubled by an injection of amino-acids into the general circulation, and yet return to normal within two or three hours. During the period required by the liver to entirely rid itself of absorbed amino-acids, their concentration in the muscles suffers no appreciable fall. From the other organs (kidney, intestine, pancreas, spleen) the absorbed

amino-acids disappear less rapidly than from the liver, but whether as slowly as from the muscles has not yet been determined. The disappearance of amino-acids from the liver is accompanied by an increase in the urea of the blood. The results have been discussed in more detail on pp. 219 and 220.

These results support the long contended view<sup>12</sup> that the liver is the organ especially responsible for the catabolism of those protein digestion products not utilized for tissue construction. The following explanation is consistent with the facts thus far ascertained. The amino-acids, with perhaps some peptides, from the intestine enter the circulation, from which they are almost immediately absorbed by the tissues. The power to take them up from the blood stream is common to all the tissues, but is limited. The muscles of the dog, for example, reach the saturation point when they contain about 75 mgm. of amino acid nitrogen per 100 grams. The liver, however, continually desaturates itself by metabolizing the amino-acids that it has absorbed, and consequently maintains indefinitely its power to continue removing them from the circulation, so long as they do not enter it faster than the liver can metabolize them. When the entrance is unnaturally rapid, as in our injection experiments, or when the liver is sufficiently degenerated, as observed clinically in some pathological conditions, the kidney assists in removing the amino-acids by excreting them unchanged. Death may result when the above agencies for preventing undue accumulation of protein digestion products are overtaxed (see Experiment 5).

In regard to the synthesis of tissue proteins, it appears reasonable to believe that, since each tissue has its own store of amino-acids, which it can replenish from the blood, it uses these to synthesize its own proteins.

<sup>12</sup> Münzer and Winterberg: *Arch. f. exp. Path. u. Pharm.*, xxxiii, p. 163.

1

## THE FATE OF PROTEIN DIGESTION PRODUCTS IN THE BODY.

### V. THE EFFECTS OF FEEDING AND FASTING ON THE AMINO-ACID CONTENT OF THE TISSUES.

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In regard to the manner in which the free amino-acids stored in the tissues are utilized we may assume two possibilities.

1. The amino-acids serve as a reserve energy supply, like glycogen, or as a reserve of tissue building material. In either case the supply would be depleted if not renewed from the food.

2. The amino-acids are merely intermediate steps in both the construction and breakdown of the tissue proteins. In this case they could originate, not only from absorbed food products, but also from autolyzed tissue protein: starvation would not result in a disappearance of the amino-acid supply of the tissues, and might even increase it.

In order to obtain evidence on the point, the tissues of dogs in various states of nutrition were analyzed for free amino-acid nitrogen by the methods described in our second paper. All the analyses were conducted in the same manner, the ammonia being removed from the extracts before the amino nitrogen was determined. The dogs were killed by bleeding. Only males were utilized, in order that possible complicating effects of pregnancy might be avoided. The results, given in the accompanying table, indicate milligrams of amino nitrogen found per 100 grams of fresh tissue. They are decisively in accord with the second of the two above explanations. The free amino-acids of the tissues do not disappear during fasting; if anything, they tend to increase.<sup>1</sup>

<sup>1</sup> Buglia and Constantino report also an increase in the "formoltitrier-bar" nitrogen (ammonia+amino-acids+amines) of the muscles during fast-

No.	1	2	3	4	5	6	7
PREVIOUS TREATMENT	MALE. REGULAR MIXED DIET TILL DAY OF DEATH. THEN RECEIVED 1 LB. FRESH BEEF FIVE HOURS BEFORE KILLED	MALE. WT. 16 KG. FED 500 GRAMS BEEF DAILY IN ADDITION TO REGULAR DIET OR SEVEN DAYS BEFORE DEATH. LAST MEAL TWENTY HOURS BEFORE KILLED	MALE. WT. 16 KG. NORMAL DIET. LAST MEAL EIGHTEEN HOURS BEFORE DEATH. CHYME STILL IN INTESTINE	MALE. WT. 12 KG. FASTED FORTY-EIGHT HOURS	MALE. WT. 10 KG. FASTED FOUR DAYS	MALE. WT. 12 KG. FASTED SIX DAYS	MALE. FASTED TWELVE DAYS. WT. BEFORE FAST, 16 KG., AFTER FAST, 12.5 KG.
Right gracilis muscle.....	66	51	57	53	46	64	60
Left gracilis muscle.....	67	52	56	54	52	58	61
Right triceps muscle.....		61	80	64	61	71	
Left triceps muscle.....		58	78	64	64	72	
Liver, lobe 1....	43	59	85	69	70	93	95
Liver, lobe 2....	44	55	86	64	68	87	85
Liver, lobe 3....		64	78	73	71	85	90
Right kidney....	40	56	64	45	84	70	85
Left kidney....		50	79	48	85	71	97
Pancreas.....	66	61	107	55	80	74	79
Spleen.....	70	92	147	69	93	99	
Duodenum.....		72	127	69	75	82	73
Jejunum.....	76	54	101	45	49	67	71
Ileum.....		54	70	36	43	74	43
Blood.....	8	6	8	6	7	5	5

The amino-acids appear, therefore, to be intermediate steps, not only in the synthesis, but in the breaking down of body proteins. Otherwise, in order to explain their maintenance in the tissues during starvation, one would be forced, contrary to the conclusions of all experimental work on the subject,<sup>2</sup> to assume that they are inert substances, lying unchanged for long periods, even

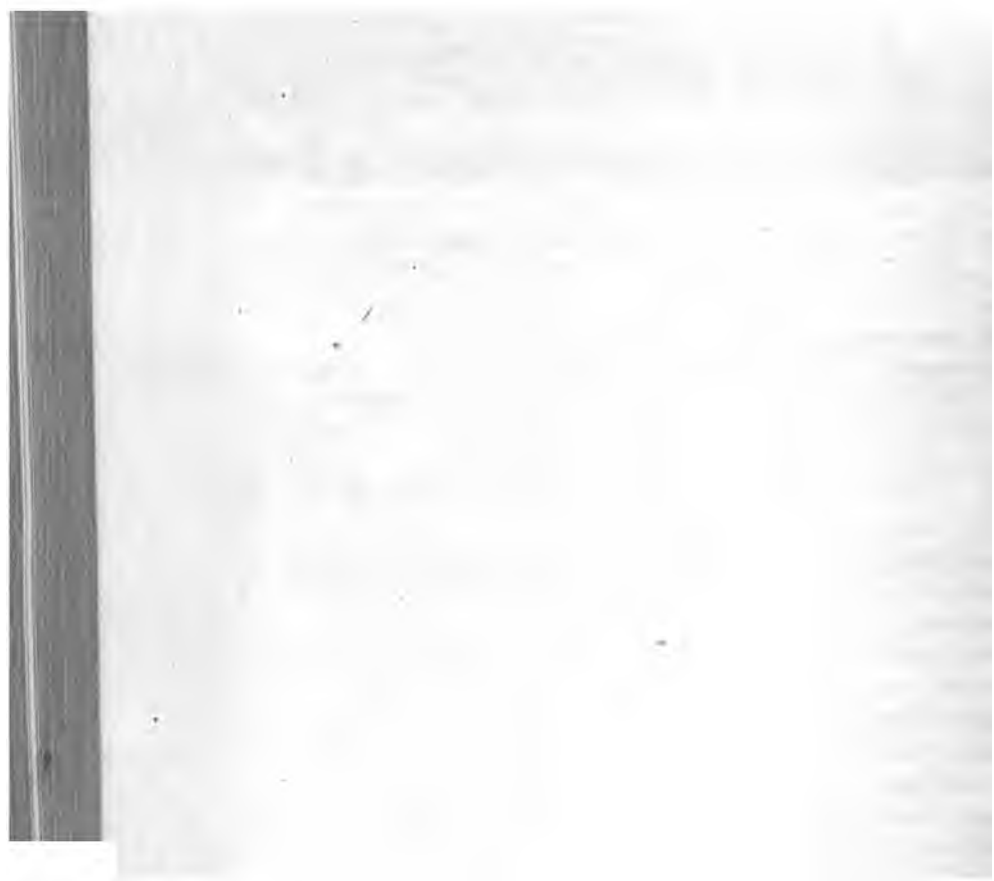
ing. They find in normal dogs 77-84 mg. "formol" nitrogen per 100 grams fresh muscle. In a dog that had fasted twelve days the figure was 95; sixteen days, 91; twenty days, 105; twenty-five days, 100.

<sup>2</sup> See articles by Levene, Kober, and Meyer cited in fourth paper.



when most urgently needed to build tissue or supply energy. The maintenance of the amino-acid supply by synthesis, from ammonia and the products of fats or carbohydrates, seems excluded. The supply of raw material in the form of fat and carbohydrates nearly disappears during starvation, and the ammonia could originate only from broken-down protein, as the normal store of ammonia nitrogen is only a fraction of that of the free amino-acids. These considerations, and the self-evident wasting of starved tissues, point strongly to autolysis as the main source of the free amino-acids in the fasting body.

The failure to increase the free amino-acid content of the tissues by high protein feeding indicates, furthermore, that when nitrogen is retained in the organism it is not, to an appreciable extent, as stored digestion products, but rather as body protein.



# THE INFLUENCE OF SALTS COMMON IN ALKALI SOILS UPON THE GROWTH OF THE RICE PLANT.

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## PART I. INFLUENCE OF SINGLE SALTS UPON THE GROWTH OF RICE SEEDLINGS.

Investigations on the influence of the salts common in alkali soils upon the growth of young seedlings have been made by many authors. In 1887, Hindolf<sup>1</sup> observed a good influence of magnesium and calcium chloride upon the early development of many cultivated plants. Coupin<sup>2</sup> studied the toxic influence of many salts upon the growth of the young root of wheat and found that calcium chloride was toxic in concentration of  $\frac{1}{100}$ . Hébert<sup>3</sup> also investigated the toxicity of chromium, aluminium and magnesium salts upon the growth of germinated seeds of wheat and rape and observed that the toxic action of magnesium

<sup>1</sup> *Jost. bot. Jahrber.*, i, p. 139, 1887.

<sup>2</sup> *Compt. rend. de l'Acad. des Sci.*, cxxxii, p. 645, 1901.

<sup>3</sup> *Bull. soc. chim. de France* (iv), i, p. 10-26, 1907; *Dietrichs' Jahrber. Agrik.-Chem.*, xi, p. 252, 1908.

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salts was least among these salts and often harmless. Since the discovery of large areas of alkali soils in western parts of the United States, the toxicity of the various salts common in alkali soils upon the growth of plants has been studied by many American authors, especially by Kearney and Harter.<sup>4</sup> Their results appear in the following table.

*Critical concentrations of pure solutions.*

SALTS USED	PLANTS TESTED								
	WHITE LUPINE		ALFALFA	WHEAT	MAIZE	SORGHUM	OATS	COTTON	BEET
	I	II							
MgSO <sub>4</sub> .....	0.00125 N	0.007 N	≈0.001 N	0.005 N	0.25 N	0.001875 N	0.001875 N	0.000312 N	0.0005 N
MgCl <sub>2</sub> .....	0.0025 N	0.0075 N	≈0.002 N	0.005 N	0.08 N	0.001875 N	0.001875 N	0.0004 N	0.0005 N
Na <sub>2</sub> CO <sub>3</sub> .....	0.005 N	0.0125 N		0.0125 N	0.015 N	0.00625 N	0.00625 N	0.005 N	0.0025 N
Na <sub>2</sub> SO <sub>4</sub> .....	0.0075 N	0.04 N		0.04 N	0.05 N	0.0175 N	0.0175 N	0.005 N	0.00675 N
NaCl.....	0.02 N	0.045 N		0.045 N	0.04 N	0.02 N	0.02 N	0.00625 N	0.025 N
NaHCO <sub>3</sub> .....	0.02 N	0.03 N		0.025 N	0.05 N	0.0075 N	0.0075 N	0.00625 N	0.0075 N

They concluded that different species differ vastly in the absolute degree of their resistance to the toxic action of these pure solutions, also the order of toxicity of the several salts varies considerably according to the species. Furthermore, the salts of magnesium are generally more toxic than those of sodium to all the plants tested with the single exception of maize.

Burlingham<sup>5</sup> has studied the influence of magnesium sulphate upon the growth of seedlings of abutilon, pea and corn, and his results were summarized as follows:

Magnesium sulphate in solutions of greater concentrations than  $\frac{M}{175}$  has a toxic action on most seedlings, the degree of toxicity varying with the type of seedlings and with the conditions. An  $\frac{M}{175}$  solution is toxic to pea seedlings, slightly stimulating to abutilon, while it has a marked stimulating effect on corn seedlings. Maximum stimulation in magnesium sulphate results in solution from  $\frac{M}{32768}$  to  $\frac{M}{131072}$ , the point again varying according to the kind of seedling grown. When magnesium sulphate is used in proper dilutions there may be produced a total growth nearly double that in the control: or in the case of abutilon seedlings, a growth of the primary root increased, but the lateral roots develop sooner, are more

<sup>4</sup>Bulletin No. 13, Bureau of Plant Industry, U. S. Dept. of Agriculture, 1907.

<sup>5</sup>Journ. Amer. Chem. Soc., xxix, pp. 1095-1112, 1907.

numerous, and attain a greater growth. Furthermore the stimulation is not limited to the root system, but the magnesium forces a more rapid and a greater growth of the hypocotyl and plumule. In this same concentration, calcium nitrate causes very little stimulation.

In addition to the marked stimulation which magnesium sulphate causes when it is used in dilutions from  $\frac{M}{15,554}$  to  $\frac{M}{334,288}$ , it increases the vitality of the seedlings. The seedlings grown in the magnesium sulphate out-lived those in the control by two or three weeks, and in some cases by a greater period.

From the foregoing results and conclusions, it is then evident that magnesium sulphate, in the absence of other salts, is not necessarily injurious in its effects, but on the other hand may be highly beneficial; while any inhibitory action is due to the presence of a relatively large proportion of magnesium in the solution.

From the preceding investigations, it will be observed that the salts act on the growth of young seedlings as toxic or stimulating agents according to their concentrations.

In regard to the influence of these salts upon the growth of the rice plant, which is the most important crop in our country, a special investigation has not been made to date. But in 1909 the widely distributed alkali soils were discovered by Prof. Dr. K. Oshima and K. Shibuya, the Chemist of the Formosa Government, in the southern part of Formosa, and now it has become a most important subject of study. We undertook this study at the suggestion of Prof. Dr. K. Oshima, in order to find out the influence of the alkali salts upon the growth of rice seedlings, and selected magnesium sulphate, magnesium chloride, calcium chloride, sodium sulphate, sodium chloride, sodium carbonate and bicarbonate as the salts to be examined.

### *Experiment I.*

In the first experiment we began with the young rice seedlings, 15-16 mm. high, which were grown in distilled water from seeds which were almost uniform in size and specific gravity (1.2-1.25). Fifty-six beakers of about 5.5 cm. diameter and 7 cm. deep, each containing 50 cc. of  $\frac{M}{2}$ ,  $\frac{M}{15}$ ,  $\frac{M}{25}$ ,  $\frac{M}{100}$ ,  $\frac{M}{250}$ ,  $\frac{M}{1000}$ ,  $\frac{M}{3000}$ ,  $\frac{M}{10000}$  solution of each salt mentioned above, were used for the experiment, the seedlings being placed in the solutions on August 3, 1911. A control experiment was carried out with distilled water. Twenty-

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SALTS USED		M 2	CONCENTRATIONS										CONTROL
			M 10	M 20	M 100	M 200	M 1000	M 2000	M 10000	M 20000	M 100000		
Magnesium sulphate	Length of leaf*	20	40	60	80	83	112	97	90	80			
	Length of root	35	65	65	80	85	140	118	100	125			
	Number of roots	1	1	1	3	3	9	6	5	6			
Magnesium chloride	Length of leaf	died	27	56	80	83	93	100	88	80			
	Length of root		42	45	70	90	165	110	112	125			
	Number of roots		1	1	2	2	6	8	6	6			
Calcium chloride	Length of leaf	died	30	47	73	80	90	90	105	80			
	Length of root		25	40	97	100	150	100	150	125			
	Number of roots		3	6	6	6	8	9	7	6			
Sodium sulphate	Length of leaf	died	33	42	120	107	100	97	93	80			
	Length of root		30	40	90	85	90	100	100	125			
	Number of roots		1	6	7	7	9	7	9	6			
Sodium chloride	Length of leaf	died	42	67	97	95	85	82	82	80			
	Length of root		30	63	150	130	70	110	130	125			
	Number of roots		3	6	5	5	4	7	7	6			
Sodium carbonate	Length of leaf	died	died	33	83	117	117	97	85	80			
	Length of root			20	30	145	145	105	85	125			
	Number of roots			1	5	5	6	7	7	6			
Sodium bicarbonate	Length of leaf	died	30	40	105	115	110	103	103	80			
	Length of root		18	45	90	140	60	90	105	125			
	Number of roots		1	1	6	5	6	6	7	6			

\* Lengths of leaf and root are expressed in millimeters.

five seedlings were grown in each culture at ordinary temperature and the evaporated water was supplemented with distilled water from time to time to keep the solutions at their original concentrations. After ten days, the difference of development was very striking, and then the determinations recorded in the table on p. 238 were made:

The results show that each salt acted as a toxic agent or a stimulant upon the growth of rice seedlings, according to its concentrations. Magnesium sulphate and chloride, calcium chloride and sodium carbonate were injurious when the concentrations were greater than about  $\frac{M}{1000}$ , while sodium sulphate, chloride and bicarbonate were toxic when the concentrations were greater than  $\frac{M}{100}$ . In every salt, when the concentration was such that the toxic action ceased, the stimulating effect began and attained its highest degree in the following order of concentration: magnesium sulphate  $\frac{M}{1000}$ , magnesium chloride  $\frac{M}{2000}$ , calcium chloride  $\frac{M}{1000}$ , sodium sulphate  $\frac{M}{100}$ , sodium chloride  $\frac{M}{100}$ , sodium carbonate  $\frac{M}{200}$  to  $\frac{M}{1000}$  and sodium bicarbonate  $\frac{M}{200}$ .

### *Experiment II.*

On June 13, 1912, twenty-five rice seeds of almost uniform size and specific gravity (1.158–1.185) were sown in the beakers, about 55 cm. in diameter and 7 cm. deep, each containing 30 cc. of solution of each salt while distilled water served as control. The concentration of the salts is indicated in the table. The beakers were kept in a room of normal temperature, and evaporated water was supplemented with distilled water from time to time to keep the solutions always in their initial concentrations. After thirty-six days, the difference of their development was very striking. The measurements recorded on the following page were then made:

It is assumed that the plant is adversely affected by the salts, if the length of root be half that of the control plants, even though the length of leaf be greater than that of the control leaf.

In this case as in the previous experiment, the growth of the seedlings was injured or stimulated by each salt according to the concentration. In the concentration at which the toxic action ceases, the stimulating action began and attained its highest point at certain definite dilutions. The growth was injured by mag-

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SALTS USED		CONCENTRATIONS									
		N 3	N 5	N 16	N 30	N 100	N 300	N 1000	N 5000	CONTROL	
Magnesium sulphate.	Length of leaf*	Only 18 seeds germinated	22	30	85	100	130	110	120	100	
	Length of root.				10	60	80	80	100	115	
	Number of roots.				1	4	6	6	6	6	
Magnesium chloride.	Length of leaf	No seed germinated	All seeds germinated but not one developed	25	83	105	100	105	107	100	
	Length of root.				30	105	100	120	143	115	
	Number of roots.				3	3	6	6	6	6	
Calcium chloride.....	Length of leaf	No seed germinated	Only 6 seeds germinated	18	85	105	110	145	95	100	
	Length of root.				40	75	90	190	130	115	
	Number of roots.				5	6	6	6	6	6	
Sodium sulphate.....	Length of leaf	No seed germinated	12	23	150	165	145	120	100	100	
	Length of root.				30	70	85	75	80	115	
	Number of roots.				4	4	6	7	7	6	
Sodium chloride.....	Length of leaf	No seed germinated	4	35	155	137	110	110	110	100	
	Length of root.				95	125	130	120	120	115	
	Number of roots.				6	6	6	7	7	6	
Sodium carbonate.....	Length of leaf	No seed germinated	Only 5 seeds germinated	5	85	120	120	110	110	100	
	Length of root.				27	40	78	100	135	115	
	Number of roots.				6	7	7	7	7	6	
Sodium bicarbonate..	Length of leaf	No seed germinated	17	55	95	120	125	100	110	100	
	Length of root.			2	105	105	105	100	100	115	
	Number of roots.			2	4	5	6	6	7	6	

\* Lengths of leaf and root are expressed in millimeters.



nesium sulphate in concentration greater than  $\frac{N}{100}$  and highly stimulated by  $\frac{N}{300}$ . Magnesium chloride was also toxic in concentration greater than  $\frac{N}{100}$  and attained highest stimulating point in concentration of  $\frac{N}{3000}$ . The toxic concentration of calcium chloride, sodium sulphate, sodium chloride, sodium carbonate and bicarbonate in each case was greater than  $\frac{N}{100}$ ,  $\frac{N}{50}$ ,  $\frac{N}{100}$ ,  $\frac{N}{100}$ ,  $\frac{N}{50}$  respectively and highest stimulation was reached in dilution of  $\frac{N}{1000}$ ,  $\frac{N}{100}$ ,  $\frac{N}{100}$ ,  $\frac{N}{300}$  and  $\frac{N}{300}$  respectively.

For convenience of comparison, the concentration of toxicity and stimulation of the seven salts in the two experiments are brought together in the following table.

SALTS USED	CONCENTRATION OF TOXICITY		DILUTION OF HIGHEST STIMULATION	
	Experiment I	Experiment II	Experiment I	Experiment II
	greater than	greater than		
Magnesium sulphate...	$\frac{M}{100}$ ( $\frac{N}{30}$ )	$\frac{N}{100}$	$\frac{M}{1000}$ ( $\frac{N}{300}$ )	$\frac{N}{300}$
Magnesium chloride...	$\frac{M}{100}$ ( $\frac{N}{50}$ )	$\frac{N}{100}$	$\frac{M}{2000}$ ( $\frac{N}{1000}$ )	$\frac{N}{3000}$
Calcium chloride.....	$\frac{M}{200}$ ( $\frac{N}{100}$ )	$\frac{N}{100}$	$\frac{M}{10000}$ ( $\frac{N}{3000}$ )	$\frac{N}{1000}$
Sodium sulphate.....	$\frac{M}{100}$ ( $\frac{N}{30}$ )	$\frac{N}{50}$	$\frac{M}{100}$ ( $\frac{N}{50}$ )	$\frac{N}{100}$
Sodium chloride.....	$\frac{M}{100}$ ( $\frac{N}{50}$ )	$\frac{N}{100}$	$\frac{M}{100}$ ( $\frac{N}{100}$ )	$\frac{N}{100}$
Sodium carbonate.....	$\frac{M}{100}$ ( $\frac{N}{30}$ )	$\frac{N}{100}$	$\frac{M}{200}$ to $\frac{M}{1000}$ ( $\frac{N}{100}$ to $\frac{N}{300}$ )	$\frac{N}{300}$
Sodium bicarbonate....	$\frac{M}{100}$ ( $\frac{N}{50}$ )	$\frac{N}{50}$	$\frac{M}{200}$ ( $\frac{N}{100}$ )	$\frac{N}{300}$

As seen in the table, both results almost coincide on the toxic and stimulating point. A slight fluctuation of these points is probably due to the fact that the plant growth varies to a certain extent with the temperature and other factors, since these experiments were not carried on at constant temperature and under identical conditions.

#### Conclusions.

From these two experiments we may safely conclude as follows:

1. The alkali salts under examination act as agents both toxic and stimulating upon the growth of rice seedlings, according to their concentrations.
2. The toxic concentrations of magnesium sulphate and chloride, calcium chloride, sodium chloride and carbonate are greater

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than  $\frac{N}{100}$  while sodium sulphate and bicarbonate are greater than  $\frac{N}{50}$ .

3. The highest stimulation is observed in the dilution of  $\frac{N}{100}$  for magnesium sulphate,  $\frac{N}{1000}$  to  $\frac{N}{3000}$  for magnesium chloride,  $\frac{N}{1000}$  to  $\frac{N}{5000}$  for calcium chloride,  $\frac{N}{50}$  to  $\frac{N}{100}$  for sodium chloride,  $\frac{N}{100}$  to  $\frac{N}{500}$  for sodium carbonate and bicarbonate.

### PART II. ON THE ANTAGONISM BETWEEN THE TOXIC EFFECTS OF TWO SALTS UPON THE GROWTH OF RICE SEEDLINGS.

The results of experiments with a single salt solution have been reported in the preceding section, but they cannot be correlated with our knowledge of alkali soils, since, as Kearney and Cameron<sup>6</sup> pointed out, in nature we have always to do with a mixture of salts and never with single solutions. They found, as in Loeb's striking experiments with marine animals, that by adding sodium salts to the solution of magnesium salts the critical concentrations of the latter could be raised considerably. In the case of *Lupinus albus* and *Medicago sativa*, the neutralizing effect became enormous when salts of calcium were added to the solutions of sulphate and chlorides of magnesium and sodium.

The physiology of the decreasing toxicity of a salt due to the presence of a second salt in the solution, was specially discussed by Osterhout<sup>7</sup> from the view-point of Loeb's conception of a "physiologically balanced salts solution." It has been shown that marine plants as well as marine animals are very sensitive to pure salt solutions, but thrive well in solutions containing a mixture of salts, even though each component is present in an amount that is toxic in pure solution. A mixture of the more important salts present in sea water, each at about the concentration at which it occurs in the sea, was found to be the best medium for the growth of marine algae. The same phenomenon has been observed in the case of land plants.

Kearney and Harter<sup>8</sup> investigated the neutralizing effect of calcium sulphate upon the toxicity of magnesium and sodium salts with eight

<sup>6</sup> Bulletin No. 71, Bureau of Plant Industry, U. S. Dept. of Agriculture, 1912.

<sup>7</sup> This Journal, i, pp. 363-369, 1906; Bot. Gaz., xlii, pp. 127-134, 1906; Univ. of Calif. Pub. Bot., ii, p. 317, 1907; Jahrb. f. wiss. Bot. xlv, p. 121, 1908; Bot. Gaz., xlv, p. 117, 1908; Univ. of Calif. Pub. Bot., iii, pp. 331-337, 1908; Bot. Gaz., xlviii, pp. 98-104, 1909.

<sup>8</sup> Bulletin No. 113, Bureau of Plant Industry, U. S. Dept. of Agriculture, 1907.

different land plants and found that the presence of calcium sulphate tends greatly to diminish, not only the differences between different species as to their tolerance of magnesium and sodium salts, but also the differences between the latter in their toxicity to the same species. The neutralizing effect of calcium sulphate is generally much more marked with magnesium than with sodium salts.

In 1907, Benecke<sup>9</sup> studied the poisonous action of various salts upon the growth of *spirogyra*. The result of his investigation was summarized as follows: Chloride, nitrate, sulphate and phosphate of sodium, potassium, magnesium and iron are more or less poisonous, and among these cations iron and magnesium are more poisonous than potassium, sodium is less poisonous than potassium; among the anions, chlorine is least poisonous. The toxicity of these anions and cations can be neutralized or decreased by the addition of calcium ions. Loew and Aso<sup>10</sup> also studied the same subject in relation to *spirogyra* and observed that calcium salts can prevent the toxic effects of magnesium salts while potassium salts can retard but not entirely prevent the injurious action of the same.

Takeuchi<sup>11</sup> has pointed out, at the end of his investigation on the behavior of algae to salts at certain concentrations, that the injurious action of magnesium salts can only completely be overcome by calcium salts, and not by sodium or potassium salts. This has been observed not only with algae, but also with young plants of barley and maize which were deprived of their endosperm.

Hansteen<sup>12</sup> has recently investigated the antagonism between cations upon the growth of wheat seedlings and shown that the pure solutions of potassium, sodium and magnesium salts are more or less injurious according to their concentrations. But in combination with calcium salts, their injurious effect on the growth of leaves, roots and root-hairs is greatly decreased.

Toxic and antagonistic effects of salts as related to ammonia formation by *Bacillus subtilis* were also investigated by Lipman<sup>13</sup> and the following conclusions were reported: 1. Each of the four chlorides ( $\text{CaCl}_2$ ,  $\text{MgCl}_2$ ,  $\text{KCl}$ ,  $\text{NaCl}$ ) is toxic for *Bacillus subtilis*, in the order given, the first being the most toxic and the fourth the least. This is different from the results with higher plants, where magnesium is the most toxic and calcium the least. 2. A marked antagonism exists between calcium and potassium, magnesium and sodium, potassium and sodium. 3. No antagonism exists between magnesium and calcium but the toxic effect of each is increased by combination with the other. This is just the opposite of what has hitherto been found for plants.

<sup>9</sup> *Ber. d. bot. Gesellsch.*, xxv, p. 322, 1907.

<sup>10</sup> *Bull. Coll. Agric.*, Tokyo Imp. Univ., vii, pp. 395-409, 1906-08.

<sup>11</sup> *Ibid.*, vii, p. 628, 1906-08.

<sup>12</sup> *Nyt. Mag. Naturvidensk.*, xlvii, pp. 181-192, 1909; ref. *Exp. Sta. Rec.*, U. S. Dept. of Agriculture, xxiii, p. 28, 1910.

<sup>13</sup> *Bot. Gaz.*, xlviii, pp. 105-124, 1909.

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As above stated, it is clear that the toxicity of a single salt solution may be neutralized by the presence of a second salt, especially calcium salts. It was desirable to investigate the influence of salts common in alkali soils upon the growth of rice plants. We have therefore selected chloride of sodium, magnesium and calcium, and sulphate of sodium and magnesium as the salts to be tested and have examined the respective antagonisms between these salts in combination.

### I. *Experiment with NaCl and MgCl<sub>2</sub>.*

The antagonism between sodium and magnesium chloride was established with young rice seedlings, about 10 mm. high, which were grown in distilled water from seeds of almost uniform size and specific gravity (1.185–1.200). Beakers of about 5.5 cm. in diameter and 7 cm. deep, each containing the solutions noted in the table<sup>14</sup> were used for the experiment, the seedlings being placed in the culture fluids on November 19 (1912). Five seedlings were grown in each culture in the greenhouse and the evaporated water was supplemented with distilled water from time to time to keep the solutions at the initial dilutions. After twelve days, the difference in development in the respective cultures was very remarkable. The following determinations were made:

SOLUTIONS USED	LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
	mm.	mm.	
$\frac{N}{10}$ NaCl, 30 cc.....	38	25	1
$\frac{N}{10}$ NaCl, 25 cc. + $\frac{N}{10}$ MgCl <sub>2</sub> , 5 cc.....	57	40	6*
$\frac{N}{10}$ NaCl, 20 cc. + $\frac{N}{10}$ MgCl <sub>2</sub> , 10 cc.....	53	30	4*
$\frac{N}{10}$ NaCl, 15 cc. + $\frac{N}{10}$ MgCl <sub>2</sub> , 15 cc.....	50	32	3*
$\frac{N}{10}$ NaCl, 10 cc. + $\frac{N}{10}$ MgCl <sub>2</sub> , 20 cc.....	45	28	2*
$\frac{N}{10}$ NaCl, 5 cc. + $\frac{N}{10}$ MgCl <sub>2</sub> , 25 cc.....	45	20	1
$\frac{N}{10}$ MgCl <sub>2</sub> , 30 cc.....	42	20	1
Distilled water, 30 cc.....	50	80	6

\*Only one root was well developed.

<sup>14</sup> As already proved in the previous section, a pure solution of each salt to be tested is very injurious to the growth of rice seedlings in the concentration of  $\frac{N}{10}$ .

From these results, it is clear that the poisonous effect of sodium and magnesium chloride largely disappears when we mix the two salts in favorable proportions. This phenomenon is due to the antagonism between sodium and magnesium ions, since the anions were similar in both salts. In these favorable mixtures, the length of leaf became greater than that in distilled water, but the length of roots and the number of roots was invariably less than in the case of the control plants. It is evident therefore that the toxic effect of sodium and of magnesium ions was mutually counteracted but not completely neutralized. And it is further evident that the toxic effect of the sodium ion was antagonized much more by magnesium ion than the latter by the former, an observation which coincides with the results of Osterhout obtained with wheat seedlings.

## II. *Experiment with Na<sub>2</sub>SO<sub>4</sub> and MgSO<sub>4</sub>.*

The antagonistic action of sodium and magnesium ions on each other was once more tested with sodium and magnesium sulphate by exactly the same method as in the preceding experiment, except that the young seedlings transplanted were about 20 mm. in height at the beginning of the experiment. The results obtained were as follows:

SOLUTIONS USED		LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
		mm.	mm.	
$\frac{N}{10}$ Na <sub>2</sub> SO <sub>4</sub> , 30 cc.		45	35	1
$\frac{N}{10}$ Na <sub>2</sub> SO <sub>4</sub> , 25 cc. + $\frac{N}{10}$ MgSO <sub>4</sub> , 5 cc.		60	40	5*
$\frac{N}{10}$ Na <sub>2</sub> SO <sub>4</sub> , 20 cc. + $\frac{N}{10}$ MgSO <sub>4</sub> , 10 cc.		55	30	5*
$\frac{N}{10}$ Na <sub>2</sub> SO <sub>4</sub> , 15 cc. + $\frac{N}{10}$ MgSO <sub>4</sub> , 15 cc.		55	35	4*
$\frac{N}{10}$ Na <sub>2</sub> SO <sub>4</sub> , 10 cc. + $\frac{N}{10}$ MgSO <sub>4</sub> , 20 cc.		55	40	3*
$\frac{N}{10}$ Na <sub>2</sub> SO <sub>4</sub> , 5 cc. + $\frac{N}{10}$ MgSO <sub>4</sub> , 25 cc.		50	33	1
$\frac{N}{10}$ MgSO <sub>4</sub> , 30 cc.		40	20	1
Distilled water, 30 cc.		80	50	7

\* Only one root was well developed.

In this case, we also observed that the mutual counteraction between sodium and magnesium ions was clearly revealed, though they did not perfectly neutralize each other. The neutralizing power of magnesium ion toward the toxic effect of sodium ion

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was greater than that of sodium to magnesium, for in the case of  $\frac{N}{10}$   $\text{Na}_2\text{SO}_4$ , 25 cc., +  $\frac{N}{10}$   $\text{MgSO}_4$ , 5 cc., the highest development of the seedlings was observed. The result of this experiment almost coincides with that of the preceding one.

### III. Experiment with $\text{NaCl}$ and $\text{CaCl}_2$ .

The antagonistic phenomenon between sodium and calcium ions was examined with  $\frac{N}{10}$  solution of sodium and calcium chloride in manner identical with that followed in the case of the experiment with sodium and magnesium chloride. Twelve days after the seedlings were transplanted to the respective culture solutions, they showed very remarkable differences of development. The plants were measured on that day with the following result.

SOLUTIONS USED	LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
	mm.	mm.	
$\frac{N}{10}$ $\text{NaCl}$ , 30 cc.....	38	25	1
$\frac{N}{10}$ $\text{NaCl}$ , 25 cc. + $\frac{N}{10}$ $\text{CaCl}_2$ , 5 cc.....	69	60	9
$\frac{N}{10}$ $\text{NaCl}$ , 20 cc. + $\frac{N}{10}$ $\text{CaCl}_2$ , 10 cc.....	55	50	7
$\frac{N}{10}$ $\text{NaCl}$ , 15 cc. + $\frac{N}{10}$ $\text{CaCl}_2$ , 15 cc.....	47	35	5
$\frac{N}{10}$ $\text{NaCl}$ , 10 cc. + $\frac{N}{10}$ $\text{CaCl}_2$ , 20 cc.....	47	40	5
$\frac{N}{10}$ $\text{NaCl}$ , 5 cc. + $\frac{N}{10}$ $\text{CaCl}_2$ , 25 cc.....	47	35	3
$\frac{N}{10}$ $\text{CaCl}_2$ , 30 cc.....	40	35	1
Distilled water, 30 cc.....	50	80	6

It is evident that in a mixture of sodium and calcium ions in proper proportion, each of which individually is poisonous, the toxic effect of these ions is almost mutually counteracted and a medium is produced in which the plant may live almost indefinitely. The toxic effect of sodium ion almost completely disappeared when we added a little calcium ion; on the other hand, the poisonous effect of calcium ion was excluded by the addition of a great amount of sodium ion.

### IV. Experiment with $\text{MgCl}_2$ and $\text{CaCl}_2$ .

On July 20, 1912, thirty seeds of rice which were almost uniform in size and specific gravity (1.185-1.200) were sown in beakers of about 5.5 cm. diameter and 7 cm. deep, each containing

the solutions noted in the table. The beakers were kept at room temperature and covered with glass plates to exclude dust and retard evaporation until the seedlings reached a height of about 15 mm. The evaporated water was supplemented with distilled water from time to time to keep the culture media at their initial concentrations. The difference in the development of the plants became very marked. On August 20, the plants were measured, and the results obtained are shown in the following table:

SOLUTIONS USED		LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
		mm.	mm.	
$\frac{N}{10}$ CaCl <sub>2</sub> , 30 cc.	.....	35	8	1
$\frac{N}{10}$ CaCl <sub>2</sub> , 25 cc. + $\frac{N}{10}$ MgCl <sub>2</sub> , 5 cc.	.....	55	5	2
$\frac{N}{10}$ CaCl <sub>2</sub> , 20 cc. + $\frac{N}{10}$ MgCl <sub>2</sub> , 10 cc.	.....	80	6	5
$\frac{N}{10}$ CaCl <sub>2</sub> , 15 cc. + $\frac{N}{10}$ MgCl <sub>2</sub> , 15 cc.	.....	95	25	4
$\frac{N}{10}$ CaCl <sub>2</sub> , 10 cc. + $\frac{N}{10}$ MgCl <sub>2</sub> , 20 cc.	.....	105	22	5
$\frac{N}{10}$ CaCl <sub>2</sub> , 5 cc. + $\frac{N}{10}$ MgCl <sub>2</sub> , 25 cc.	.....	110	40	8
$\frac{N}{10}$ MgCl <sub>2</sub> , 30 cc.	.....	45	5	1

From the above table, it is clear that in a mixture of calcium and magnesium ions the toxic effects of these cations were mutually counteracted. The amount of calcium required to antagonize the toxic effect of magnesium was less than that of the latter to the former, for we observed that the highest development of the plants was attained in the mixture of  $\frac{N}{10}$  CaCl<sub>2</sub>, 5 cc., +  $\frac{N}{10}$  MgCl<sub>2</sub>, 25 cc.; consequently the antagonizing power of calcium is strong and that of magnesium is weak.

#### V. Experiment with NaCl and Na<sub>2</sub>SO<sub>4</sub>.

In the above four experiments, we examined the antagonisms between the metallic ions in regard to their toxic effects upon the growth of rice seedlings. We then undertook to investigate the question of the mutual power of counteracting injurious effects of anions upon the development of rice plants. Hence, sodium chloride and sulphate were selected as salts to be tested and examined in a manner similar to that followed in the case of the experiment with sodium and magnesium chloride. The plants were measured as follows:

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SOLUTIONS USED	LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
	mm.	mm.	
$\frac{N}{10}$ NaCl, 30 cc.....	42	30	1
$\frac{N}{10}$ NaCl, 25 cc. + $\frac{N}{10}$ Na <sub>2</sub> SO <sub>4</sub> , 5 cc.....	60	40	1
$\frac{N}{10}$ NaCl, 20 cc. + $\frac{N}{10}$ Na <sub>2</sub> SO <sub>4</sub> , 10 cc.....	53	30	1
$\frac{N}{10}$ NaCl, 15 cc. + $\frac{N}{10}$ Na <sub>2</sub> SO <sub>4</sub> , 15 cc.....	42	30	1
$\frac{N}{10}$ NaCl, 10 cc. + $\frac{N}{10}$ Na <sub>2</sub> SO <sub>4</sub> , 20 cc.....	52	30	1
$\frac{N}{10}$ NaCl, 5 cc. + $\frac{N}{10}$ Na <sub>2</sub> SO <sub>4</sub> , 25 cc.....	55	30	1
$\frac{N}{10}$ Na <sub>2</sub> SO <sub>4</sub> , 30 cc.....	45	35	1
Distilled water, 30 cc.....	80	50	7

The counteraction observed in this experiment is doubtless due to the actions between the anions (Cl' and SO<sub>4</sub>'') present in the culture media since the cations in both salts are the same. The ratio of these anions required to produce the most favorable medium for the development of the plants was 25:5, although the development of the seedling did not reach that of the control plants. The antagonistic power of the SO<sub>4</sub>'' ion required to neutralize the toxic effect of Cl' ion was slightly greater than that of Cl' to the SO<sub>4</sub>'' ion.

### VI. Experiment with MgCl<sub>2</sub> and MgSO<sub>4</sub>.

The antagonism between Cl' and SO<sub>4</sub>'' ions was again examined with magnesium chloride and sulphate in the same manner as in the preceding experiment. The following result which is similar to that of the experiment with sodium chloride and sulphate was obtained.

SOLUTIONS USED	LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
	mm.	mm.	
$\frac{N}{10}$ MgCl <sub>2</sub> , 30 cc.....	45	35	1
$\frac{N}{10}$ MgCl <sub>2</sub> , 25 cc. + $\frac{N}{10}$ MgSO <sub>4</sub> , 5 cc.....	60	40	1
$\frac{N}{10}$ MgCl <sub>2</sub> , 20 cc. + $\frac{N}{10}$ MgSO <sub>4</sub> , 10 cc.....	55	30	1
$\frac{N}{10}$ MgCl <sub>2</sub> , 15 cc. + $\frac{N}{10}$ MgSO <sub>4</sub> , 15 cc.....	48	30	1
$\frac{N}{10}$ MgCl <sub>2</sub> , 10 cc. + $\frac{N}{10}$ MgSO <sub>4</sub> , 20 cc.....	50	25	1
$\frac{N}{10}$ MgCl <sub>2</sub> , 5 cc. + $\frac{N}{10}$ MgSO <sub>4</sub> , 25 cc.....	55	25	1
$\frac{N}{10}$ MgSO <sub>4</sub> , 30 cc.....	40	25	1
Distilled water, 30 cc.....	80	50	7



VII. *Experiment with NaCl and MgSO<sub>4</sub>.*

The antagonistic action of Na<sup>+</sup>, Mg<sup>++</sup>, Cl<sup>-</sup>, and SO<sub>4</sub><sup>''</sup> ions on each other was established with sodium chloride and magnesium sulphate in the same manner as in the case of Experiment I.

The following result was obtained.

SOLUTIONS USED		LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
		mm.	mm.	
$\frac{N}{10}$ NaCl, 30 cc.....		38	25	1
$\frac{N}{10}$ NaCl, 25 cc. + $\frac{N}{10}$ MgSO <sub>4</sub> , 5 cc.....		55	37	6*
$\frac{N}{10}$ NaCl, 20 cc. + $\frac{N}{10}$ MgSO <sub>4</sub> , 10 cc.....		50	33	4*
$\frac{N}{10}$ NaCl, 15 cc. + $\frac{N}{10}$ MgSO <sub>4</sub> , 15 cc.....		45	30	3*
$\frac{N}{10}$ NaCl, 10 cc. + $\frac{N}{10}$ MgSO <sub>4</sub> , 20 cc.....		48	31	1
$\frac{N}{10}$ NaCl, 5 cc. + $\frac{N}{10}$ MgSO <sub>4</sub> , 25 cc.....		48	32	1
$\frac{N}{10}$ MgSO <sub>4</sub> , 30 cc.....		30	25	1
Distilled water, 30 cc.....		50	80	6

\* Only one root was well developed.

From the table, it is clear that the observed antagonistic action between these ions almost coincides with the results of Experiments I and V or VI.

VIII. *Experiment with Na<sub>2</sub>SO<sub>4</sub> and MgCl<sub>2</sub>.*

The same antagonism as in the preceding experiment was again examined with sodium sulphate and magnesium chloride as before. The result obtained almost coincides with that of the preceding experiment as will be seen in the following table.

SOLUTIONS USED		LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
		mm.	mm.	
$\frac{N}{10}$ Na <sub>2</sub> SO <sub>4</sub> , 30 cc.....		45	35	1
$\frac{N}{10}$ Na <sub>2</sub> SO <sub>4</sub> , 25 cc. + $\frac{N}{10}$ MgCl <sub>2</sub> , 5 cc.....		80	40	6*
$\frac{N}{10}$ Na <sub>2</sub> SO <sub>4</sub> , 20 cc. + $\frac{N}{10}$ MgCl <sub>2</sub> , 10 cc.....		80	38	6*
$\frac{N}{10}$ Na <sub>2</sub> SO <sub>4</sub> , 15 cc. + $\frac{N}{10}$ MgCl <sub>2</sub> , 15 cc.....		65	45	4*
$\frac{N}{10}$ Na <sub>2</sub> SO <sub>4</sub> , 10 cc. + $\frac{N}{10}$ MgCl <sub>2</sub> , 20 cc.....		50	30	1
$\frac{N}{10}$ Na <sub>2</sub> SO <sub>4</sub> , 5 cc. + $\frac{N}{10}$ MgCl <sub>2</sub> , 25 cc.....		60	25	1
$\frac{N}{10}$ MgCl <sub>2</sub> , 30 cc.....		55	40	1
Distilled water, 30 cc.....		80	50	7

\* Only one root was well developed.

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### IX. Experiment with $\text{Na}_2\text{SO}_4$ and $\text{CaCl}_2$ .

The antagonistic action of  $\text{Na}^+$ ,  $\text{Ca}^{++}$ ,  $\text{Cl}^-$  and  $\text{SO}_4^{--}$  on each other was examined with sodium sulphate and calcium chloride in the same manner as in Experiment II. The result obtained was as follows:

SOLUTIONS USED	LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
	mm.	mm.	
$\frac{N}{10}$ $\text{Na}_2\text{SO}_4$ , 30 cc.....	45	35	1
$\frac{N}{10}$ $\text{Na}_2\text{SO}_4$ , 25 cc. + $\frac{N}{10}$ $\text{CaCl}_2$ , 5 cc.....	80	90	9
$\frac{N}{10}$ $\text{Na}_2\text{SO}_4$ , 20 cc. + $\frac{N}{10}$ $\text{CaCl}_2$ , 10 cc.....	80	90	7
$\frac{N}{10}$ $\text{Na}_2\text{SO}_4$ , 15 cc. + $\frac{N}{10}$ $\text{CaCl}_2$ , 15 cc.....	70	50	7
$\frac{N}{10}$ $\text{Na}_2\text{SO}_4$ , 10 cc. + $\frac{N}{10}$ $\text{CaCl}_2$ , 20 cc.....	57	30	5
$\frac{N}{10}$ $\text{Na}_2\text{SO}_4$ , 5 cc. + $\frac{N}{10}$ $\text{CaCl}_2$ , 25 cc.....	50	38	4
$\frac{N}{10}$ $\text{CaCl}_2$ , 30 cc.....	45	25	1
Distilled water, 30 cc.....	80	50	7

As will be seen in the above result, in a suitable mixture of  $\text{Na}^+$ ,  $\text{Ca}^{++}$ ,  $\text{Cl}^-$  and  $\text{SO}_4^{--}$  ions, their toxic effects completely disappear. It was also observed that the combined antagonistic actions of cations and anions have a more favorable effect than that of one of them.

### X. Experiment with $\text{MgSO}_4$ and $\text{CaCl}_2$ .

The antagonistic action of  $\text{Mg}^{++}$ ,  $\text{Ca}^{++}$ ,  $\text{Cl}^-$  and  $\text{SO}_4^{--}$  ions on each other was established with magnesium sulphate and calcium chloride as in Experiment IV. The following result which is similar to that of the preceding experiment was obtained.

SOLUTIONS USED	LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
	mm.	mm.	
$\frac{N}{10}$ $\text{MgSO}_4$ , 30 cc.....	50	23	1
$\frac{N}{10}$ $\text{MgSO}_4$ , 25 cc. + $\frac{N}{10}$ $\text{CaCl}_2$ , 5 cc.....	135	55	4
$\frac{N}{10}$ $\text{MgSO}_4$ , 20 cc. + $\frac{N}{10}$ $\text{CaCl}_2$ , 10 cc.....	135	50	6
$\frac{N}{10}$ $\text{MgSO}_4$ , 15 cc. + $\frac{N}{10}$ $\text{CaCl}_2$ , 15 cc.....	120	45	6
$\frac{N}{10}$ $\text{MgSO}_4$ , 10 cc. + $\frac{N}{10}$ $\text{CaCl}_2$ , 20 cc.....	85	25	4
$\frac{N}{10}$ $\text{MgSO}_4$ , 5 cc. + $\frac{N}{10}$ $\text{CaCl}_2$ , 25 cc.....	80	18	3
$\frac{N}{10}$ $\text{CaCl}_2$ , 30 cc.....	35	8	1

*Conclusions.*

The results obtained in all of these experiments, may be summarized as follows:

1. The salts under examination, used separately, are very poisonous in  $\frac{N}{10}$  concentration upon the growth of the rice plant, but when the two salts are mixed with each other in a suitable proportion, the toxic effect of each salt more or less completely disappears. This result is of great importance in alkali soil investigations.

2. The antagonistic action of salts is due to that of the ions formed by the dissociation of the salt.

3. In general, divalent cations are markedly antagonized by monovalent cations, but on the other hand, monovalent cations do not strongly antagonize divalent cations.

4. Among the divalent cations, calcium shows a more marked antagonism than magnesium.

5. The antagonism between  $\text{Cl}'$  and  $\text{SO}_4''$ , though it is small in comparison with that between cations, is also present in no slight degree.

PART III. ON THE ANTAGONISTIC ACTION OF SODIUM AND  
POTASSIUM SALTS.

In Part III we have specially undertaken to test the antagonism between sodium salts, potassium salts, and sodium and potassium salts. Chloride, sulphate and nitrate of soda and potash were selected as the salts to be tested, and the following experiments were made:

I. *Experiment with  $\text{Na}_2\text{SO}_4$  and  $\text{K}_2\text{SO}_4$ .*

Eight beakers of about 5.5 cm. in diameter and 7 cm. deep each containing 30 cc. of culture fluids, of the composition noted in the table, served for the experiment. One beaker containing distilled water served as control. On February 1, 1913, young rice seedlings which were grown in distilled water, were transplanted into the beakers, each receiving five healthy individuals of uniform size (about 20 mm. long) and kept in a greenhouse.

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The evaporated water was replaced with distilled water from time to time. A decided difference in plant growth was noticed from day to day. On February 18, measurements were made with the following result:

SOLUTIONS USED		LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
		mm.	mm.	
$\frac{N}{10}$	Na <sub>2</sub> SO <sub>4</sub> , 30 cc.....	35	35	2*
$\frac{N}{10}$	Na <sub>2</sub> SO <sub>4</sub> , 25 cc. + $\frac{N}{10}$ K <sub>2</sub> SO <sub>4</sub> , 5 cc.....	67	43	6*
$\frac{N}{10}$	Na <sub>2</sub> SO <sub>4</sub> , 20 cc. + $\frac{N}{10}$ K <sub>2</sub> SO <sub>4</sub> , 10 cc.....	47	37	6*
$\frac{N}{10}$	Na <sub>2</sub> SO <sub>4</sub> , 15 cc. + $\frac{N}{10}$ K <sub>2</sub> SO <sub>4</sub> , 15 cc.....	47	37	6*
$\frac{N}{10}$	Na <sub>2</sub> SO <sub>4</sub> , 10 cc. + $\frac{N}{10}$ K <sub>2</sub> SO <sub>4</sub> , 20 cc.....	47	35	6*
$\frac{N}{10}$	Na <sub>2</sub> SO <sub>4</sub> , 5 cc. + $\frac{N}{10}$ K <sub>2</sub> SO <sub>4</sub> , 25 cc.....	50	43	7*
$\frac{N}{10}$	K <sub>2</sub> SO <sub>4</sub> , 30 cc.....	40	37	6*
	Distilled water, 30 cc.....	68	43	7

\* Only one root was well developed.

### II. Experiment with NaCl and KCl.

The antagonistic action of sodium and potassium on each other was again established with sodium and potassium chloride in the same manner as in the preceding experiment:

SOLUTIONS USED		LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
		mm.	mm.	
$\frac{N}{10}$	NaCl, 30 cc.....	35	35	2*
$\frac{N}{10}$	NaCl, 25 cc. + $\frac{N}{10}$ KCl, 5 cc.....	60	45	6*
$\frac{N}{10}$	NaCl, 20 cc. + $\frac{N}{10}$ KCl, 10 cc.....	55	45	6*
$\frac{N}{10}$	NaCl, 15 cc. + $\frac{N}{10}$ KCl, 15 cc.....	55	40	6*
$\frac{N}{10}$	NaCl, 10 cc. + $\frac{N}{10}$ KCl, 20 cc.....	42	45	7*
$\frac{N}{10}$	NaCl, 5 cc. + $\frac{N}{10}$ KCl, 25 cc.....	50	35	6*
$\frac{N}{10}$	KCl, 30 cc.....	40	25	6*
	Distilled water, 30 cc.....	68	43	7

\* Only one root was well developed.

### III. Experiment with NaNO<sub>3</sub> and KNO<sub>3</sub>.

The antagonistic action of sodium and potassium on each other was once more tested with sodium and potassium nitrate and the following result was obtained:

SOLUTIONS USED		LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
		mm.	mm.	
$\frac{N}{10}$	$\text{NaNO}_3$ , 30 cc.....	35	35	2*
$\frac{N}{10}$	$\text{NaNO}_3$ , 25 cc. + $\frac{N}{10}$ $\text{KNO}_3$ , 5 cc.....	60	37	7*
$\frac{N}{10}$	$\text{NaNO}_3$ , 20 cc. + $\frac{N}{10}$ $\text{KNO}_3$ , 10 cc.....	50	40	6*
$\frac{N}{10}$	$\text{NaNO}_3$ , 15 cc. + $\frac{N}{10}$ $\text{KNO}_3$ , 15 cc.....	50	35	7*
$\frac{N}{10}$	$\text{NaNO}_3$ , 10 cc. + $\frac{N}{10}$ $\text{KNO}_3$ , 20 cc.....	43	35	7*
$\frac{N}{10}$	$\text{NaNO}_3$ , 5 cc. + $\frac{N}{10}$ $\text{KNO}_3$ , 25 cc.....	50	35	7*
$\frac{N}{10}$	$\text{KNO}_3$ , 30 cc.....	40	35	4*
	Distilled water, 30 cc.....	68	43	7

\* Only one root was well developed.

#### IV. Experiment with $\text{K}_2\text{SO}_4$ and KCl.

The antagonism between  $\text{SO}_4^{''}$  and  $\text{Cl}'$  ions was already observed in Experiment No. V of Part II with sodium sulphate and chloride. This was then once more examined with potassium sulphate and chloride in the same manner as in Experiment I, and the result, which coincides with that of the experiment with sodium salts, is given in the following table:

SOLUTIONS USED		LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
		mm.	mm.	
$\frac{N}{10}$	$\text{K}_2\text{SO}_4$ , 30 cc.....	40	35	6*
$\frac{N}{10}$	$\text{K}_2\text{SO}_4$ , 25 cc. + $\frac{N}{10}$ KCl, 5 cc.....	47	43	7*
$\frac{N}{10}$	$\text{K}_2\text{SO}_4$ , 20 cc. + $\frac{N}{10}$ KCl, 10 cc.....	47	35	6*
$\frac{N}{10}$	$\text{K}_2\text{SO}_4$ , 15 cc. + $\frac{N}{10}$ KCl, 15 cc.....	40	32	6*
$\frac{N}{10}$	$\text{K}_2\text{SO}_4$ , 10 cc. + $\frac{N}{10}$ KCl, 20 cc.....	50	50	7*
$\frac{N}{10}$	$\text{K}_2\text{SO}_4$ , 5 cc. + $\frac{N}{10}$ KCl, 25 cc.....	47	35	7*
$\frac{N}{10}$	KCl, 30 cc.....	35	25	6*
	Distilled water, 30 cc.....	68	43	7

\* Only one root was well developed.

#### V. Experiment with $\text{Na}_2\text{SO}_4$ and $\text{NaNO}_3$ .

The antagonism between  $\text{SO}_4^{''}$  and  $\text{NO}_3'$  ions on each other was established with sodium sulphate and nitrate as in Experiment I. The following result was obtained:

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SOLUTIONS USED		LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
		mm.	mm.	
$\frac{N}{10}$ Na <sub>2</sub> SO <sub>4</sub> , 30 cc.....		35	35	2*
$\frac{N}{10}$ Na <sub>2</sub> SO <sub>4</sub> , 25 cc. + $\frac{N}{10}$ NaNO <sub>3</sub> , 5 cc.....		52	35	5*
$\frac{N}{10}$ Na <sub>2</sub> SO <sub>4</sub> , 20 cc. + $\frac{N}{10}$ NaNO <sub>3</sub> , 10 cc.....		52	35	5*
$\frac{N}{10}$ Na <sub>2</sub> SO <sub>4</sub> , 15 cc. + $\frac{N}{10}$ NaNO <sub>3</sub> , 15 cc.....		40	35	5*
$\frac{N}{10}$ Na <sub>2</sub> SO <sub>4</sub> , 10 cc. + $\frac{N}{10}$ NaNO <sub>3</sub> , 20 cc.....		50	35	5*
$\frac{N}{10}$ Na <sub>2</sub> SO <sub>4</sub> , 5 cc. + $\frac{N}{10}$ NaNO <sub>3</sub> , 25 cc.....		45	30	5*
$\frac{N}{10}$ NaNO <sub>3</sub> , 30 cc.....		35	35	2*
Distilled water, 30 cc.....		68	43	7

\* Only one root was well developed.

### VI. Experiment with K<sub>2</sub>SO<sub>4</sub> and KNO<sub>3</sub>.

An experiment similar to the preceding one was made with potassium salts and a similar result was obtained, as will be seen in the following table:

SOLUTIONS USED		LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
		mm.	mm.	
$\frac{N}{10}$ K <sub>2</sub> SO <sub>4</sub> , 30 cc.....		40	35	6*
$\frac{N}{10}$ K <sub>2</sub> SO <sub>4</sub> , 25 cc. + $\frac{N}{10}$ KNO <sub>3</sub> , 5 cc.....		58	45	7*
$\frac{N}{10}$ K <sub>2</sub> SO <sub>4</sub> , 20 cc. + $\frac{N}{10}$ KNO <sub>3</sub> , 10 cc.....		53	38	6*
$\frac{N}{10}$ K <sub>2</sub> SO <sub>4</sub> , 15 cc. + $\frac{N}{10}$ KNO <sub>3</sub> , 15 cc.....		45	38	6*
$\frac{N}{10}$ K <sub>2</sub> SO <sub>4</sub> , 10 cc. + $\frac{N}{10}$ KNO <sub>3</sub> , 20 cc.....		50	42	6*
$\frac{N}{10}$ K <sub>2</sub> SO <sub>4</sub> , 5 cc. + $\frac{N}{10}$ KNO <sub>3</sub> , 25 cc.....		40	35	6*
$\frac{N}{10}$ KNO <sub>3</sub> , 30 cc.....		40	35	4*
Distilled water, 30 cc.....		68	43	7

\* Only one root was well developed.

### VII. Experiment with NaNO<sub>3</sub> and NaCl.

The antagonistic action of NO<sub>3</sub>' and Cl' ions on each other was examined with the solution of sodium nitrate and chloride in the same manner as in the case of Experiment I. The transplanting of young rice seedlings, about 25 mm. high, took place on February 20 (1913) and the plants were measured on March 5 with the following result:

SOLUTIONS USED	LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
	mm.	mm.	
$\frac{N}{10}$ NaNO <sub>3</sub> , 30 cc.....	43	20	1
$\frac{N}{10}$ NaNO <sub>3</sub> , 25 cc. + $\frac{N}{10}$ NaCl, 5 cc.....	63	35	7*
$\frac{N}{10}$ NaNO <sub>3</sub> , 20 cc. + $\frac{N}{10}$ NaCl, 10 cc.....	59	30	6*
$\frac{N}{10}$ NaNO <sub>3</sub> , 15 cc. + $\frac{N}{10}$ NaCl, 15 cc.....	55	35	6*
$\frac{N}{10}$ NaNO <sub>3</sub> , 10 cc. + $\frac{N}{10}$ NaCl, 20 cc.....	50	25	5*
$\frac{N}{10}$ NaNO <sub>3</sub> , 5 cc. + $\frac{N}{10}$ NaCl, 25 cc.....	57	30	6*
$\frac{N}{10}$ NaCl, 30 cc.....	46	25	3*
Distilled water, 30 cc.....	78	58	7

\* Only one root was well developed.

### VIII. Experiment with KNO<sub>3</sub> and KCl.

An experiment similar to the preceding one was made with potassium salts. The result obtained was as follows:

SOLUTIONS USED	LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
	mm.	mm.	
$\frac{N}{10}$ KNO <sub>3</sub> , 30 cc.....	45	25	3*
$\frac{N}{10}$ KNO <sub>3</sub> , 25 cc. + $\frac{N}{10}$ KCl, 5 cc.....	60	35	6*
$\frac{N}{10}$ KNO <sub>3</sub> , 20 cc. + $\frac{N}{10}$ KCl, 10 cc.....	50	35	6*
$\frac{N}{10}$ KNO <sub>3</sub> , 15 cc. + $\frac{N}{10}$ KCl, 15 cc.....	48	30	6*
$\frac{N}{10}$ KNO <sub>3</sub> , 10 cc. + $\frac{N}{10}$ KCl, 20 cc.....	50	40	7*
$\frac{N}{10}$ KNO <sub>3</sub> , 5 cc. + $\frac{N}{10}$ KCl, 25 cc.....	72	40	7*
$\frac{N}{10}$ KCl, 30 cc.....	50	25	7*
Distilled water, 30 cc.....	78	58	7

\* Only one root was well developed.

### IX. Experiment with K<sub>2</sub>SO<sub>4</sub> and NaCl.

The antagonism between K<sup>+</sup>, Na<sup>+</sup>, SO<sub>4</sub><sup>''</sup> and Cl<sup>'</sup> ions on each other was established with potassium sulphate and sodium chloride according to a method similar to that of Experiment VII. The following result was obtained:

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SOLUTIONS USED	LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
	mm.	mm.	
$\frac{N}{10}$ $K_2SO_4$ , 30 cc.....	47	35	7*
$\frac{N}{10}$ $K_2SO_4$ , 25 cc. + $\frac{N}{10}$ NaCl, 5 cc.....	68	35	7*
$\frac{N}{10}$ $K_2SO_4$ , 20 cc. + $\frac{N}{10}$ NaCl, 10 cc.....	59	35	6*
$\frac{N}{10}$ $K_2SO_4$ , 15 cc. + $\frac{N}{10}$ NaCl, 15 cc.....	52	30	7*
$\frac{N}{10}$ $K_2SO_4$ , 10 cc. + $\frac{N}{10}$ NaCl, 20 cc.....	60	33	7*
$\frac{N}{10}$ $K_2SO_4$ , 5 cc. + $\frac{N}{10}$ NaCl, 25 cc.....	65	35	6*
$\frac{N}{10}$ NaCl, 30 cc.....	46	25	3*
Distilled water, 30 cc.....	78	58	7

\* Only one root was well developed.

### X. Experiment with $Na_2SO_4$ and KCl.

A similar experiment to the preceding one was made with sodium sulphate and potassium chloride. The following result which coincides with that of the previous experiment, was obtained:

SOLUTIONS USED	LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
	mm.	mm.	
$\frac{N}{10}$ $Na_2SO_4$ , 30 cc.....	45	20	2*
$\frac{N}{10}$ $Na_2SO_4$ , 25 cc. + $\frac{N}{10}$ KCl, 5 cc.....	72	25	7*
$\frac{N}{10}$ $Na_2SO_4$ , 20 cc. + $\frac{N}{10}$ KCl, 10 cc.....	65	26	7*
$\frac{N}{10}$ $Na_2SO_4$ , 15 cc. + $\frac{N}{10}$ KCl, 15 cc.....	60	18	6*
$\frac{N}{10}$ $Na_2SO_4$ , 10 cc. + $\frac{N}{10}$ KCl, 20 cc.....	64	25	7*
$\frac{N}{10}$ $Na_2SO_4$ , 5 cc. + $\frac{N}{10}$ KCl, 25 cc.....	71	28	5*
$\frac{N}{10}$ KCl, 30 cc.....	50	25	7*
Distilled water, 30 cc.....	78	58	7

\* Only one root was well developed.

### XI. Experiment with $Na_2SO_4$ and $KNO_3$ .

The antagonistic action of  $Na^+$ ,  $K^+$ ,  $SO_4^{''}$  and  $NO_3'$  ions on each other was examined with sodium sulphate and potassium nitrate in the same manner as in Experiment VII. The result obtained was as follows:



SOLUTIONS USED	LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
	mm.	mm.	
$\frac{N}{10}$ $\text{Na}_2\text{SO}_4$ , 30 cc. ....	45	20	2*
$\frac{N}{10}$ $\text{Na}_2\text{SO}_4$ , 25 cc. + $\frac{N}{10}$ $\text{KNO}_3$ , 5 cc. ....	65	35	6*
$\frac{N}{10}$ $\text{Na}_2\text{SO}_4$ , 20 cc. + $\frac{N}{10}$ $\text{KNO}_3$ , 10 cc. ....	70	30	7*
$\frac{N}{10}$ $\text{Na}_2\text{SO}_4$ , 15 cc. + $\frac{N}{10}$ $\text{KNO}_3$ , 15 cc. ....	60	30	3*
$\frac{N}{10}$ $\text{Na}_2\text{SO}_4$ , 10 cc. + $\frac{N}{10}$ $\text{KNO}_3$ , 20 cc. ....	77	30	7*
$\frac{N}{10}$ $\text{Na}_2\text{SO}_4$ , 5 cc. + $\frac{N}{10}$ $\text{KNO}_3$ , 25 cc. ....	60	35	4*
$\frac{N}{10}$ $\text{KNO}_3$ , 30 cc. ....	45	25	3*
Distilled water, 30 cc. ....	78	58	7

\* Only one root was well developed.

### XII. Experiment with $\text{K}_2\text{SO}_4$ and $\text{NaNO}_3$ .

The same antagonism as in the preceding experiment was again observed with potassium sulphate and sodium nitrate. A result similar to that of Experiment XI was obtained as will be seen in the following table:

SOLUTIONS USED	LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
	mm.	mm.	
$\frac{N}{10}$ $\text{K}_2\text{SO}_4$ , 30 cc. ....	47	35	7*
$\frac{N}{10}$ $\text{K}_2\text{SO}_4$ , 25 cc. + $\frac{N}{10}$ $\text{NaNO}_3$ , 5 cc. ....	73	30	7*
$\frac{N}{10}$ $\text{K}_2\text{SO}_4$ , 20 cc. + $\frac{N}{10}$ $\text{NaNO}_3$ , 10 cc. ....	60	20	5*
$\frac{N}{10}$ $\text{K}_2\text{SO}_4$ , 15 cc. + $\frac{N}{10}$ $\text{NaNO}_3$ , 15 cc. ....	60	25	5*
$\frac{N}{10}$ $\text{K}_2\text{SO}_4$ , 10 cc. + $\frac{N}{10}$ $\text{NaNO}_3$ , 20 cc. ....	60	35	6*
$\frac{N}{10}$ $\text{K}_2\text{SO}_4$ , 5 cc. + $\frac{N}{10}$ $\text{NaNO}_3$ , 25 cc. ....	72	40	5*
$\frac{N}{10}$ $\text{NaNO}_3$ , 30 cc. ....	43	20	1
Distilled water, 30 cc. ....	78	58	7

\* Only one root was well developed.

### XIII. Experiment with $\text{KNO}_3$ and $\text{NaCl}$ .

The antagonism between  $\text{K}^+$ ,  $\text{Na}^+$ ,  $\text{NO}_3^-$  and  $\text{Cl}^-$  ions in combination with each other was examined with potassium nitrate and sodium chloride in a manner similar to that of Experiment VII. The following result was obtained:

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SOLUTIONS USED	LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
	mm.	mm.	
$\frac{N}{10}$ KNO <sub>3</sub> , 30 cc.....	45	25	3*
$\frac{N}{10}$ KNO <sub>3</sub> , 25 cc. + $\frac{N}{10}$ NaCl, 5 cc.....	65	30	5*
$\frac{N}{10}$ KNO <sub>3</sub> , 20 cc. + $\frac{N}{10}$ NaCl, 10 cc.....	62	30	5*
$\frac{N}{10}$ KNO <sub>3</sub> , 15 cc. + $\frac{N}{10}$ NaCl, 15 cc.....	60	25	4*
$\frac{N}{10}$ KNO <sub>3</sub> , 10 cc. + $\frac{N}{10}$ NaCl, 20 cc.....	63	25	5*
$\frac{N}{10}$ KNO <sub>3</sub> , 5 cc. + $\frac{N}{10}$ NaCl, 25 cc.....	75	35	7*
$\frac{N}{10}$ NaCl, 30 cc.....	46	25	1
Distilled water, 30 cc.....	78	58	7

\* Only one root was well developed.

### XIV. Experiment with NaNO<sub>3</sub> and KCl.

The antagonistic phenomenon observed in Experiment XIII was again tested with sodium nitrate and potassium chloride. The following result, which almost coincides with that of the preceding experiment, was obtained:

SOLUTIONS USED	LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
	mm.	mm.	
$\frac{N}{10}$ NaNO <sub>3</sub> , 30 cc.....	43	30	1
$\frac{N}{10}$ NaNO <sub>3</sub> , 25 cc. + $\frac{N}{10}$ KCl, 5 cc.....	75	30	7*
$\frac{N}{10}$ NaNO <sub>3</sub> , 20 cc. + $\frac{N}{10}$ KCl, 10 cc.....	65	35	6*
$\frac{N}{10}$ NaNO <sub>3</sub> , 15 cc. + $\frac{N}{10}$ KCl, 15 cc.....	62	35	6*
$\frac{N}{10}$ NaNO <sub>3</sub> , 10 cc. + $\frac{N}{10}$ KCl, 20 cc.....	64	25	4*
$\frac{N}{10}$ NaNO <sub>3</sub> , 5 cc. + $\frac{N}{10}$ KCl, 25 cc.....	70	25	7*
$\frac{N}{10}$ KCl, 30 cc.....	50	25	7*
Distilled water, 30 cc.....	78	58	7

\* Only one root was well developed.

### Conclusions.

From the above results we may conclude as follows:

1. Sodium and potassium salts are antagonized by each other. The curve of antagonism between these salts shows two maxima and the location of these maxima is almost constant, occurring at the point of the proportion of 5:25. This coincides with the result which was observed by Osterhout<sup>15</sup> on wheat seedlings.
2. The antagonism between these salts is due to cations as well as anions.

<sup>15</sup> Bot. Gaz., xlviii, pp. 98-104, 1909.

3. The antagonism between anions is small in comparison with that between cations.

#### PART IV. ON THE ANTAGONISM BETWEEN POTASSIUM AND MAGNESIUM OR CALCIUM IONS.

The antagonism between potassium and magnesium or calcium ions is especially interesting as shown in the experiment of Osterhout<sup>16</sup> on wheat seedlings and of Loeb<sup>17</sup> on *Fundulus*. In these experiments, it was shown that the toxicity of potassium ion is antagonized by magnesium or calcium ions, though calcium shows a more marked antagonism than magnesium. We have undertaken to investigate this relation in the case of growing rice seedlings, and accordingly we have made the following experiments:

##### I. *Experiment with KCl and MgCl<sub>2</sub>.*

The antagonism between potassium and magnesium ions was established with the young seedlings of rice, about 25 mm. high, which were grown in distilled water from seeds of almost uniform size and specific gravity (1.185–1.200). Beakers of about 5.5 cm. in diameter and 7 cm. deep, each containing 30 cc. of the culture fluids, were used for the experiment. The seedlings were placed in the solutions on March 7, 1913. Five seedlings were grown in each culture in the greenhouse and the evaporated water was supplemented with distilled water from time to time so as to keep the solutions at their initial dilutions. On March 24, the difference in development in the respective cultures was very striking, and the following determinations were made:

SOLUTIONS USED	LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
	mm.	mm.	
$\frac{N}{10}$ KCl, 30 cc.....	40	17	3
$\frac{N}{10}$ KCl, 25 cc. + $\frac{N}{10}$ MgCl <sub>2</sub> , 5 cc.....	60	16	4
$\frac{N}{10}$ KCl, 20 cc. + $\frac{N}{10}$ MgCl <sub>2</sub> , 10 cc.....	58	15	6
$\frac{N}{10}$ KCl, 15 cc. + $\frac{N}{10}$ MgCl <sub>2</sub> , 15 cc.....	55	15	7
$\frac{N}{10}$ KCl, 10 cc. + $\frac{N}{10}$ MgCl <sub>2</sub> , 20 cc.....	55	15	5
$\frac{N}{10}$ KCl, 5 cc. + $\frac{N}{10}$ MgCl <sub>2</sub> , 25 cc.....	56	21	4
$\frac{N}{10}$ MgCl <sub>2</sub> , 30 cc.....	43	23	3
Distilled water, 30 cc.....	63	47	11

<sup>16</sup> *Bot. Gaz.*, xlv, p. 117, 1908; xlviii, pp. 98–104, 1909.

<sup>17</sup> *Amer. Journ. of Physiol.*, iii, p. 327, 1900.

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In pure magnesium chloride solution the seedlings had grown only 18 mm. in eighteen days; in potassium chloride solution, only 15 mm.; while in distilled water the length of leaf had attained to 63 mm. Therefore, it is evident that potassium chloride and magnesium chloride have a poisonous action upon the growth of rice seedlings.

This poisonous effect largely disappears when we mix the two salts ( $\text{MgCl}_2 + \text{KCl}$ ) in proper proportions. In the mixture  $\frac{N}{10}$  KCl, 25 cc. +  $\frac{N}{10}$   $\text{MgCl}_2$ , 5 cc., the growth of the seedlings was most vigorous and their height had reached to 60 mm. Therefore, it is evident that in the mixture of magnesium and potassium chloride in favorable proportion, the seedlings grow about twice as much as in pure solutions.

It will be noticed that decreasing the proportion of potassium or increasing the amount of magnesium beyond the optimum proportion causes unfavorable conditions for the growth of the seedlings. Accordingly, it is inferred that a small amount of magnesium retards the toxic effect of potassium, and on the other hand, potassium retards the injurious action of magnesium in large amount.

### II. Experiment with KCl and $\text{CaCl}_2$ .

The antagonistic action of potassium and calcium ions on each other was examined with potassium and calcium chloride in the same manner as in the first experiment. The result obtained was as follows:

SOLUTIONS USED	LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
	mm.	mm.	
$\frac{N}{10}$ KCl, 30 cc.....	40	17	3
$\frac{N}{10}$ KCl, 25 cc. + $\frac{N}{10}$ $\text{CaCl}_2$ , 5 cc.....	68	51	10
$\frac{N}{10}$ KCl, 20 cc. + $\frac{N}{10}$ $\text{CaCl}_2$ , 10 cc.....	65	25	8
$\frac{N}{10}$ KCl, 15 cc. + $\frac{N}{10}$ $\text{CaCl}_2$ , 15 cc.....	65	25	8
$\frac{N}{10}$ KCl, 10 cc. + $\frac{N}{10}$ $\text{CaCl}_2$ , 20 cc.....	65	25	8
$\frac{N}{10}$ KCl, 5 cc. + $\frac{N}{10}$ $\text{CaCl}_2$ , 25 cc.....	64	20	8
$\frac{N}{10}$ $\text{CaCl}_2$ , 30 cc.....	35	18	3
Distilled water, 30 cc.....	63	47	11

The result obtained was similar to that of the previous experiment, but it is clear that calcium has a more marked antagonistic action than magnesium and decidedly prevents the toxicity of the potassium ion.

*Conclusion.*

Potassium and magnesium or calcium salts are poisonous to the rice plant when used separately but when mixed together in suitable proportion the poisonous effect more or less completely disappears. The results coincide with those of Osterhout and form an important factor in the question of soil fertility.

PART V. CAN BARIUM AND STRONTIUM REPLACE THE  
ANTAGONISTIC ACTION OF CALCIUM?

It has been pointed out that the injurious action of certain metallic ions upon the growth of rice seedlings may be perfectly neutralized by the presence of calcium ions. It was of interest to experiment with barium and strontium, which are similar to calcium in chemical properties, to determine whether they exert an action similar to that of calcium. In order to investigate this problem we have used sodium and magnesium chloride as the toxic salts for the following experiments.

I. *Experiment with  $MgCl_2$ .*

Twenty beakers of about 5.5 cm. diameter and 7 cm. deep, served for the experiment. While one beaker which contained 30 cc. of distilled water served as check, the other nineteen beakers received the solutions noted in the table. Five seedlings, about 25 mm. high, which were grown in distilled water from seeds of almost uniform size and specific gravity (1.185–1.200), were transplanted in each of the respective beakers on February 25, 1913, and kept in the greenhouse. The evaporated water was supplemented with distilled water from time to time so as to keep the culture solutions at their initial concentration. On March 14, the difference in development in the respective cultures was very striking, and measurements were then made with the following result:

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SOLUTIONS USED	LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
	mm.	mm.	
Distilled water, 30 cc.....	68	60	8
$\frac{N}{10}$ MgCl <sub>2</sub> , 30 cc.....	53	12	1
$\frac{N}{10}$ MgCl <sub>2</sub> , 25 cc. + $\frac{N}{10}$ CaCl <sub>2</sub> , 5 cc.....	68	50	9
$\frac{N}{10}$ MgCl <sub>2</sub> , 20 cc + $\frac{N}{10}$ CaCl <sub>2</sub> , 10 cc.....	65	40	10
$\frac{N}{10}$ MgCl <sub>2</sub> , 15 cc. + $\frac{N}{10}$ CaCl <sub>2</sub> , 15 cc.....	62	35	8
$\frac{N}{10}$ MgCl <sub>2</sub> , 10 cc. + $\frac{N}{10}$ CaCl <sub>2</sub> , 20 cc.....	60	20	8
$\frac{N}{10}$ MgCl <sub>2</sub> , 5 cc. + $\frac{N}{10}$ CaCl <sub>2</sub> , 25 cc.....	52	15	6
$\frac{N}{10}$ CaCl <sub>2</sub> , 30 cc.....	44	12	8
$\frac{N}{10}$ MgCl <sub>2</sub> , 25 cc + $\frac{N}{10}$ BaCl <sub>2</sub> , 5 cc.....	40	12	1
$\frac{N}{10}$ MgCl <sub>2</sub> , 20 cc. + $\frac{N}{10}$ BaCl <sub>2</sub> , 10 cc.....	33	10	1
$\frac{N}{10}$ MgCl <sub>2</sub> , 15 cc. + $\frac{N}{10}$ BaCl <sub>2</sub> , 15 cc.....	28	8	1
$\frac{N}{10}$ MgCl <sub>2</sub> , 10 cc. + $\frac{N}{10}$ BaCl <sub>2</sub> , 20 cc.....	28	7	1
$\frac{N}{10}$ MgCl <sub>2</sub> , 5 cc. + $\frac{N}{10}$ BaCl <sub>2</sub> , 25 cc.....	28	5	1
$\frac{N}{10}$ BaCl <sub>2</sub> , 30 cc.....	24	8	1
$\frac{N}{10}$ MgCl <sub>2</sub> , 25 cc. + $\frac{N}{10}$ SrCl <sub>2</sub> , 5 cc.....	60	12	3
$\frac{N}{10}$ MgCl <sub>2</sub> , 20 cc. + $\frac{N}{10}$ SrCl <sub>2</sub> , 10 cc.....	45	8	3
$\frac{N}{10}$ MgCl <sub>2</sub> , 15 cc. + $\frac{N}{10}$ SrCl <sub>2</sub> , 15 cc.....	40	5	2
$\frac{N}{10}$ MgCl <sub>2</sub> , 10 cc. + $\frac{N}{10}$ SrCl <sub>2</sub> , 20 cc.....	35	7	1
$\frac{N}{10}$ MgCl <sub>2</sub> , 5 cc. + $\frac{N}{10}$ SrCl <sub>2</sub> , 25 cc.....	30	9	1
$\frac{N}{10}$ SrCl <sub>2</sub> , 30 cc.....	22	8	1

The result shows that the presence of calcium in proper proportion can exert only a beneficial action, while in the case of barium, on the contrary, a depression resulted. Although strontium in suitable proportion retarded the toxic action of magnesium, it is far inferior to calcium.

### II. Experiment with NaCl.

Twenty beakers, each containing 30 cc. of culture fluids, served for the experiment. The culture solutions were applied in the same proportion as in Experiment I using NaCl instead of MgCl<sub>2</sub>.

Five seedlings, about 20 mm. high, were transplanted on March 7, 1913, and kept in the greenhouse. The evaporated water was supplemented with distilled water from time to time. The plants had developed very well with remarkable differences in growth. The plants were measured on March 24 with the following result which coincides with that of the preceding experiment.

SOLUTIONS USED	LENGTH OF LEAF	LENGTH OF ROOT	NUMBER OF ROOTS
	mm.	mm.	
Distilled water, 30 cc.....	65	50	9
$\frac{N}{10}$ NaCl, 30 cc.....	44	13	1
$\frac{N}{10}$ NaCl, 25 cc. + $\frac{N}{10}$ CaCl <sub>2</sub> , 5 cc.....	70	40	9
$\frac{N}{10}$ NaCl, 20 cc. + $\frac{N}{10}$ CaCl <sub>2</sub> , 10 cc.....	70	40	9
$\frac{N}{10}$ NaCl, 15 cc. + $\frac{N}{10}$ CaCl <sub>2</sub> , 15 cc.....	60	25	8
$\frac{N}{10}$ NaCl, 10 cc. + $\frac{N}{10}$ CaCl <sub>2</sub> , 20 cc.....	56	20	8
$\frac{N}{10}$ NaCl, 5 cc. + $\frac{N}{10}$ CaCl <sub>2</sub> , 25 cc.....	50	20	6
$\frac{N}{10}$ CaCl <sub>2</sub> , 30 cc.....	44	22	6
$\frac{N}{10}$ NaCl, 25 cc. + $\frac{N}{10}$ BaCl <sub>2</sub> , 5 cc.....	40	20	3
$\frac{N}{10}$ NaCl, 20 cc. + $\frac{N}{10}$ BaCl <sub>2</sub> , 10 cc.....	41	18	5
$\frac{N}{10}$ NaCl, 15 cc. + $\frac{N}{10}$ BaCl <sub>2</sub> , 15 cc.....	30	15	3
$\frac{N}{10}$ NaCl, 10 cc. + $\frac{N}{10}$ BaCl <sub>2</sub> , 20 cc.....	30	20	3
$\frac{N}{10}$ NaCl, 5 cc. + $\frac{N}{10}$ BaCl <sub>2</sub> , 25 cc.....	28	17	3
$\frac{N}{10}$ BaCl <sub>2</sub> , 30 cc.....	29	10	3
$\frac{N}{10}$ NaCl, 25 cc. + $\frac{N}{10}$ SrCl <sub>2</sub> , 5 cc.....	50	22	6
$\frac{N}{10}$ NaCl, 20 cc. + $\frac{N}{10}$ SrCl <sub>2</sub> , 10 cc.....	47	20	4
$\frac{N}{10}$ NaCl, 15 cc. + $\frac{N}{10}$ SrCl <sub>2</sub> , 15 cc.....	45	16	5
$\frac{N}{10}$ NaCl, 10 cc. + $\frac{N}{10}$ SrCl <sub>2</sub> , 20 cc.....	40	10	4
$\frac{N}{10}$ NaCl, 5 cc. + $\frac{N}{10}$ SrCl <sub>2</sub> , 25 cc.....	36	16	4
$\frac{N}{10}$ SrCl <sub>2</sub> , 30 cc.....	28	15	3

### Conclusion.

The injurious effect of certain metallic ions upon the growth of rice seedlings may be perfectly counteracted only by the presence of calcium ions. Strontium ions can exert an influence only slightly retarding the toxicity of the metallic ions. Barium ion not only has no beneficial action, but a depressing effect is observed. Consequently, it is concluded that barium and strontium cannot replace the antagonistic action of calcium.





## THE DETERMINATION OF OXYBUTYRIC ACID.<sup>1</sup>

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(Received for publication, September 4, 1913.)

In 1908 one of us described a method<sup>2</sup> for the determination of oxybutyric acid in urine, based upon its oxidation by chromic acid with the formation of acetone, the latter being determined by standard iodine and thiosulphate solutions in the usual way. The procedure was combined with the determination of acetone, preformed and from diacetic acid, so that the technique as described accomplished the determination of the three "acetone bodies" in the same sample of urine and with practically the same reagents.

The advantages of this method for the determination of oxybutyric acid over those based upon the optical activity of the extracted acid are believed to be the shorter time required; the fact that even very small as well as large amounts may be determined with the same degree of accuracy; and the combined determination of the related "acetone bodies" in the same sample of urine in one procedure.

At the time this method was described it was believed that practically theoretical results were obtained when the specified conditions were adhered to. This belief was based on a series of determinations on a solution of the inactive acid which had been purified by recrystallization of the sodium salt, the titrated acidity being taken as the criterion of the concentration. These results varied

<sup>1</sup> A part of the work described in this paper was done during the summers of 1911 and 1912 in the Biochemical Laboratory of the Harvard Medical School. My thanks are due Professor Folin for placing the facilities of his laboratory at my disposal. I am indebted also to Professor Christian and to Dr. Joslin of Harvard, to Dr. I. Greenwald of the Chemical Laboratory of the Montefiore Home in New York, and to Doctor Jesse Myer of Washington University, for a supply of diabetic urine.—P. A. S.

<sup>2</sup> Shaffer: *This Journal*, v, p. 211, 1908.

from 98.8 to 103.3 per cent of the theoretical values, and were accepted as showing that the oxidation and formation of acetone is practically a quantitative reaction. None of the known salts of oxybutyric acid proved suitable to use as a standard, and the levo acid was not used for the purpose because of an inability to get it to crystallize and of a lack of confidence at the time in its published specific rotation.

Gorslin and Cooke<sup>3</sup> have suggested slight modifications in the method but did not question its accuracy. Mondschein<sup>4</sup> also has published figures indicating that he obtained satisfactory results. It now appears, however, that the claim of theoretical results for the method is not altogether correct.

Embden and Schmitz,<sup>5</sup> in criticizing the method, state that the results are probably somewhat too low since they obtained only a fraction of the optical value when the raw extracts prepared from urines for the polarization method were oxidized by bichromate. With urines containing much of the acid 80 to 90 per cent or more of the optical values were obtained on oxidizing the extracts, while the extracts from urines containing little of the acid gave on oxidation only a small part of the optical value. Embden and Schmitz accordingly believe that the ether extracts of such urines contain levo-rotary substances other than the oxybutyric acid and that the optical values are therefore too high, while the results of the oxidation method are somewhat too low. They suggest that the true results lie between the values obtained by the polariscopic and oxidation methods.

These findings together with our intention of using the method for other work involving the determination of oxybutyric acid and acetone in blood and tissues, for which there existed no suitable procedure, led us to undertake a review of the various optical points of the oxidation method and of the method based upon the activity of the ether extracts.

A series of determinations by the oxidation method on solutions of  $\beta$ -oxybutyric acid as extracted from the urine by ether (Black's method) gave results which confirmed the statement of Embden

<sup>3</sup> Gorslin and Cooke: this *Journal*, x, p. 291, 1911-12.

<sup>4</sup> Mondschein: *Biochem. Zeitschr.*, xlii, p. 95, 1912.

<sup>5</sup> Embden and Schmitz: *Handbuch der biochemischen Arbeitsmethoden*, 1910, Bd. iii, p. 934.

and Schmitz and show that the optical values of such extracts are greater than those obtained by oxidation.

SOLUTION	OPTICAL VALUE	OXIDATION METHOD	PER CENT OF OPTICAL VALUE
	<i>grams in 100 cc.</i>		
1. From diabetic urine..... ( $\alpha = -14.84^\circ$ , $l = 2.2$ $[\alpha]_D^{20} = -24.12^\circ$ )	28.0	26.4 25.75 26.1 25.95 25.67 25.83 25.33	94.5 92.0 93.3 92.7 91.8 92.5 90.5
Average.....		25.86	92.4
2. From diabetic urine..... ( $\alpha = 1.92^\circ$ , $l = 2.$ )	3.98	3.40 3.42	85.5 86.0
3. From non-diabetic urine..... ( $\alpha = -0.76^\circ$ , $l = 2.$ )	1.576	1.265	80.3
4. From non-diabetic urine..... ( $\alpha = -0.83^\circ$ , $l = 2.$ )	1.72	1.44	84.0
5. From non-diabetic urine..... ( $\alpha = -1.69^\circ$ , $l = 2.$ )	3.50	2.97 3.07 3.05	85.0 87.6 87.2
6. From diabetic urine.....	2.06	2.05	98.6
7. From diabetic urine.....	2.10	2.05	97.8
8. From diabetic urine.....	2.04	1.95	95.5
9. From diabetic urine.....	2.06	1.92	93.3
10. From normal urine <sup>6</sup> .....	0.58	0.21	48.0

According to these results the values obtained on oxidation of the urine extracts vary from about 48 per cent of the optical value in the case of the extract from normal urine (the greater part of which was not oxybutyric acid) to about 98 per cent, usually between 85 and 95 per cent. These findings led to a somewhat

<sup>6</sup> The same solution after precipitation by basic lead acetate and ammonia gave by the oxidation method values equivalent to 0.058 per cent  $\beta$ -oxybutyric acid, or only 10 per cent of the original optical value; 90 per cent of the levo-rotary substance extracted from this urine was therefore not oxybutyric acid. Oxybutyric acid is not precipitated when its solutions are treated with basic lead acetate and ammonia. Occasionally it may appear that there is a loss of 1 to 2 per cent, but such amounts are within the limits of accuracy of which the method is ordinarily capable, and are negligible. The following example shows the difference sometimes observed: 0.5885 gram of pure calcium zinc oxybutyrate was dissolved and made up

lengthy study of the factors which might explain the discrepancies. In order first to test whether the oxidation method as carried out actually yields low results, it was necessary to carry out a series of determinations, on solutions of synthetic and *l*-oxybutyric acid of known purity. Since the methods of preparation and purification differ in some particulars from those heretofore used, the details are given below.

*Purification of  $\beta$ -oxybutyric acid.*

The impure *l*-acid as extracted from urine, especially after fermentation of the sugar, invariably is contaminated with other organic acids. For the removal of these Magnus-Levy<sup>7</sup> has suggested the neutralization of the extract with calcium carbonate and the addition of an equal volume of alcohol to the solution of calcium salts, under which conditions he claims the salts of most of the other acids separate, the calcium oxybutyrate remaining in solution. This is not in accordance with our experience; as a rule one gets no precipitation. Calcium lactate and the salts of whatever other acids that may be present are quite soluble in 50 per cent or even much stronger alcohol. If, however, instead of calcium the acids are converted into the zinc salts and alcohol added, the lactate and perhaps also other salts are fairly completely precipitated on standing, while the zinc oxybutyrate, quite contrary to statements in the literature, is very soluble in both alcohol and water, and remains in solution. We have used this procedure. After filtering off the precipitated zinc salts the alcohol is boiled off, the

to 250 cc. 100-cc. portions of this solution were (1) treated with basic lead acetate and ammonia, diluted to 250 cc. and filtered; and (2) diluted to 250 cc. and filtered. Determinations were made by the oxidation method in 75-cc. portions of each filtrate, equivalent to 30 cc. of the original solution which contained 0.0706 gram salt or 0.0567 gram oxybutyric acid.

	Mgm. acetone found	Oxybutyric acid	Per cent of theory (wt.)
Without lead.....	29.1	52.2	92.0
	29.2	52.4	92.4
	29.0	52.0	91.8
After lead precipitation.....	28.4	51.0	90.0
	28.3	50.8	89.5
	28.6	51.3	90.5

<sup>7</sup> Magnus-Levy: *Ergeb. d. inn. Med. u. Kinderheilk.*, i, p. 414, 1908.

syrup cooled, strongly acidified with sulphuric acid (50 per cent) and plaster or anhydrous sodium sulphate added and the mixture allowed to harden. If the syrup is much colored, pure bone black may be added before the plaster. The coarsely powdered material is then extracted with ether in a Soxhlet apparatus. After removing the ether from the extract the residue is dissolved in 10 parts or less of water and if necessary shaken cold with a little pure bone black and filtered.

The inactive acid is conveniently made according to Wislicenus<sup>8</sup> by the reduction of aceto-acetic ester by sodium amalgam. The free acid is best isolated by extraction with ether after evaporating, acidifying and dehydrating the solution of the salt with plaster or sodium sulphate.

For the further purification of the acid, the salts hitherto used, with the exception of the sodium salt which is very deliquescent, have in our hands not proved suitable. Repeated and varied efforts to get the *l*-acid to crystallize have so far not been successful. A new double salt of calcium and zinc was, however, discovered which has been of considerable service. This salt, which is quite stable, crystallizes in long needles or needle-like plates, and while soluble in about 10 parts of water (*dl*-salt in 7 parts) is much less soluble than the other salts of the acid with which we have worked. The salt is prepared as follows:

*Calcium-zinc oxybutyrate.* Equal parts of the free acid are neutralized by warming with zinc carbonate and calcium carbonate respectively, the solutions filtered and poured together. Both the calcium and the zinc salts are very soluble, but when mixed, the double salt crystallizes almost at once if the concentration is greater than 10 per cent (14 per cent in the case of the *dl*-salt). The greater part of that remaining in solution is precipitated beautifully crystalline after a few hours on adding an equal volume of hot alcohol. It may be repeatedly crystallized by precipitation by alcohol, though the final crystallization should be from water by evaporation, because the alcohol causes a slight hydrolysis and the precipitation also of a little zinc hydroxide which remains undissolved when these preparations are again dissolved in water. After recrystallizing several times from water, the preparations are practically pure. The free acid may be recovered by acidify-

<sup>8</sup> Wislicenus: *Ann. d. Chem.*, cxlix, p. 205, 1869.

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ing the solutions of the salt, setting with sodium sulphate<sup>9</sup> extraction with ether.

Determinations of the specific rotation of the recrystallized calcium zinc  $\beta$ -oxybutyrate in 3 per cent to 9 per cent solution gave the average value:  $[\alpha]_D^{20} = -16.26^\circ$

PREPARATION I (a). Five times recrystallized from alcohol and once from water.

0.8742 gram in 10 cc.  $l=2.2$ ,  $\alpha = -3.12^\circ$

$$[\alpha]_D^{20} = -16.25^\circ$$

PREPARATION I (b). After again recrystallizing from water.

2.9646 gram in 50 cc.  $l=2.2$ ,  $\alpha = -2.10^\circ$

$$[\alpha]_D^{20} = -16.15^\circ$$

PREPARATION II (a). Recrystallized once from alcohol and twice from water.

3.9630 grams in 50 cc.  $l=2.2$ ,  $\alpha = -2.84^\circ$

$$[\alpha]_D^{20} = -16.28^\circ$$

PREPARATION II (b). After again recrystallizing from water.

3.7584 grams in 50 cc.  $l=2.2$ ,  $\alpha = -2.69^\circ$

$$[\alpha]_D^{20} = -16.26^\circ$$

Analysis of this salt for ash ( $\text{CaO} + \text{ZnO}$ ) and calcium corresponds to the formula  $\text{CaZn}(\text{C}_4\text{H}_7\text{O}_3)_4$ .

Ash: Cautiously ignited to constant weight with small portions of ammonium nitrate.

I. 1.2279 grams substance = 0.3243 gram ash = 26.41 per cent.

II. 0.5194 gram substance = 0.1386 gram ash = 26.68 per cent.

III. 0.7163 gram substance = 0.1898 gram ash = 26.50 per cent.

Average found = 26.53 per cent. Theory = 26.55 per cent.

Calcium determination by precipitation as oxalate after removal of zinc by hydrogen sulphide gave the following results:

I. 1.3014 grams salt = 0.3661 gram calcium oxalate ( $\text{Ca}(\text{COO})_2 + \text{H}_2\text{O}$ ) = 7.77 per cent calcium.

II. 1.3550 grams salt = 0.3837 gram calcium oxalate = 7.76 per cent calcium.

III. 1.2817 grams salt = 0.3648 gram calcium oxalate = 7.79 per cent calcium.

Theory = 7.74 per cent.

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<sup>9</sup> Powdered anhydrous sodium sulphate frequently contains small amounts of material soluble in ether which appear in the extracts. This substance may be removed by several recrystallizations of sodium sulphate from water.

The salt melts with decomposition and not sharply at about 240°C.

Determinations by the oxidation method on solutions of the above preparations of *l*-double salt and on solutions of the free acid obtained therefrom gave the following results.

For the determinations the contents of the distilling flask containing the oxybutyric acid was diluted to about 600 cc., 30 cc. of sulphuric acid (sp. gr. 1.59) added, and a total of about 0.5 gram of  $K_2Cr_2O_7$  in very dilute solution dropped in during the distillation which was continued about three and one-half hours. The acetone in the distillates was titrated with iodine and thiosulphate solutions.

I (a). 25 cc. of a solution containing 0.0874 gram salt = 0.0703 gram acid were taken for each determination.

Grams acetone found	Grams oxybutyric acid	Per cent of theory
0.0358	0.0642	91.4
0.0364	0.0653	93.0
0.0356	0.0639	90.9
0.0356	0.0639	90.9

I (b). 50 cc. of a solution containing 0.0886 gram double salt = 0.0712 gram acid were used for each determination, which was carried out as above.

Grams acetone found	Grams oxybutyric acid	Per cent of theory
0.0372	0.0668	93.8
0.0373	0.0670	94.1
0.0371	0.0665	93.4

I (c). Preparation I (b) again recrystallized from water. 0.0885 gram salt = 0.0711 gram acid was taken for each determination and carried out as above.

Grams acetone found	Grams oxybutyric acid	Per cent of theory
0.0373	0.0669	94.2
0.0370	0.0664	93.4

II (a). 0.0887 gram salt = 0.0713 gram acid taken for each determination.

Grams acetone found	Grams oxybutyric acid	Per cent of theory
0.0367	0.0658	92.5
0.0367	0.0658	92.5
0.0367	0.0658	92.5

II (b). 0.0884 gram salt = 0.0711 gram acid taken for each determination.

Grams acetone found	Grams oxybutyric acid	Per cent of theory
0.0367	0.0658	92.6
0.0367	0.0658	92.6
0.0368	0.0660	92.8
0.0366	0.0656	92.3

II (c). A solution of the *l*-acid isolated from preparation II (b).

## Determination of Oxybutyric Acid

OPTICAL VALUE:  $([\alpha]_D^{20} = -24.12^\circ) = 4.45$  per cent.

Acetone found in 50 cc. of a dilution equivalent to 1 cc. of original solution containing 0.0445 gram acid (optical value):

Grams acetone found	Grams oxybutyric acid	Per cent of theory (optical value)
0.0232	0.0416	93.5
0.0230	0.0412	92.7
0.0228	0.0409	92.0

III. *l*-Double salt three times recrystallized from water. 25 cc. containing 0.0730 gram salt = 0.0586 gram acid was used for each determination carried out as above.

Grams acetone found	Grams oxybutyric acid	Per cent of theory (weight of salt)
0.0299	0.0536	91.5
0.0305	0.0547	93.3
0.0297	0.0533	91.0
0.0299	0.0536	91.5

IV. Synthetic *dl*-calcium zinc salt, recrystallized three times from water. 50 cc. solution containing 0.08912 gram salt = 0.0715 gram acid taken for each determination.

Grams acetone found	Grams oxybutyric acid	Per cent of theory
0.0371	0.0665	93.0
0.0370	0.0663	92.8
0.0375	0.0672	94.0

The results by the oxidation method upon the supposedly pure preparations point to the conclusion that the method as carried out yields values from 5 to 10 per cent less than the theory. We have made many efforts to find conditions which would give theoretical results, but so far without complete success. Other oxidizing agents have not proved suitable; raising the temperature by inert salts has proved futile; and the substitution of other acids for sulphuric, or changes in the concentration of acid, do not give higher results. The low results are probably to be explained by a portion of the aceto-acetic acid undergoing the well-known acid decomposition, with the formation of acetic acid instead of acetone. On boiling solutions of pure oxybutyric acid or its salts in 5 per cent sulphuric acid the distillates contain little or no acid, but if a large excess of bichromate is added, considerable amounts of acid other than carbonic (about 50 cc.  $\frac{N}{10}$  in some experiments from 1 gram of calcium zinc salt) pass over, and under these conditions acetic acid is readily recognized in the distillate. It is therefore probable that small amounts of acetic acid are thus formed even



when the bichromate is added very slowly, and that even under the best conditions this decomposition amounts to about 5 per cent of the oxybutyric acid present.

It is not likely that there is a decomposition of acetone once it is formed, for experiments show that acetone is unaffected under the conditions and passes into the distillate very rapidly. Nor does the trouble lie in the titration of the acetone (see page 283).

Somewhat higher results can be obtained by adding the bichromate *very* slowly and continuing the distillation for a correspondingly long period; and conversely, as was pointed out in the first paper, if the bichromate is added too rapidly, very low results are found, and as noted above considerable amounts of acetic acid are formed. The following determinations were made as described on page 271, except that instead of 0.4 to 0.6 gram  $K_2Cr_2O_7$ , only 90 mgm. were added in very dilute solution during the first two and three-quarter hours of distillation and another 90 mgm. during a subsequent hour, the distillation being continued with slow boiling for four hours. The amount of bichromate added was still more than twice that theoretically required to oxidize the oxybutyric acid to aceto-acetic acid. The results are expressed as oxybutyric acid:

	CALCULATED FROM WT. OF SALT	AVERAGE OF THREE DETERMINATIONS AS USUALLY CARRIED OUT. 0.4-0.6 GM. $K_2Cr_2O_7$		BY SLOW OXIDATION. 0.18 GM. $K_2Cr_2O_7$	
		grams	per cent	grams	per cent
I.....	0.0711	0.0667	93.7	0.068	95.7
II.....	0.0713	0.0657	92.3	0.0696	97.5
III.....	0.0715	0.0667	93.5	0.0694	97.0
IV.....	0.0711			0.0676	95.2
V.....	0.0445	0.0412	92.7	0.0425	95.5

It is possible that still slower addition of bichromate and still longer distillation would yield nearly 100 per cent, but the accuracy of the method would not thereby be increased because when carried out as originally described, the results are practically constant, though it now appears that they represent only 90 per cent to 95 per cent of the true values. It seems preferable to retain the original directions and to add a correction of about 10 per cent to the result.

*The oxidation method as applied to urine.*

At the time of the first attempts to apply the oxidation of oxybutyric acid to its determination in urine it was found that several substances which normally or occasionally are present, interfere with the results by yielding products which use up iodine when the distillates are titrated. The substances considered were glucuronic acids, sugar, lactic, butyric and formic acids, perhaps leucine,<sup>10</sup> phenols and some unidentified substances. The possibility of any material interference by these substances was effectively obviated by the introduction of three modifications; glucuronic acids and sugar are removed by preliminary precipitation with basic lead acetate and ammonia; phenol, butyric and formic acid if present are removed during the first distillation of the acidified filtrate, which also removes the acetone (preformed and from aceto-acetic acid), the latter being titrated after redistillation from alkali. Lactic acid if present is in part converted into acetic aldehyde on oxidation with bichromate, and to obviate possible interference from this source the distillate containing it and the acetone derived from the oxybutyric acid is redistilled from alkali and hydrogen peroxide, which completely holds back as acetate the small amounts of aldehyde which may have been formed. The effect of the unidentified substances is almost wholly removed by the precipitation with lead and the final redistillation with hydrogen peroxide.

The chief criticism of the method advanced by Embden and Schmitz<sup>11</sup> is that sugar, which of course is usually present in urines in which it is desired to determine oxybutyric acid, gives rise on oxidation with bichromate to substances which use iodine when the distillates are titrated and that, contrary to the finding of one of us, this interfering product is not removed on redistilling with alkali and hydrogen peroxide, and that therefore the method cannot be applied directly to urines containing sugar. We have confirmed this statement to the extent that glucose, when oxidized with sulphuric acid and bichromate, does give (as was pointed out

<sup>10</sup> Later experiments have shown that leucine does not yield acetone when boiled with sulphuric acid and bichromate under the conditions of the method.

<sup>11</sup> *Loc. cit.*

in the first paper)<sup>12</sup> small amounts of a volatile substance which reacts with hypiodate, and that it is frequently not wholly removed by alkali and hydrogen peroxide, which was first believed to be the case. The following experiment illustrates this behavior:

2 grams glucose in 600 cc. water plus 10 cc. concentrated  $\text{H}_2\text{SO}_4$  were distilled, dropping in 3 per cent  $\text{K}_2\text{Cr}_2\text{O}_7$ . The distillate was redistilled after adding 30 cc. of 3 per cent  $\text{H}_2\text{O}_2$  and 5 cc. of 10 per cent  $\text{NaOH}$ .

	cc. $\frac{N}{10}$ iodine used
2 grams glucose.....	5.2
Duplicate.....	4.3
1 gram glucose.....	0.6
Duplicate.....	2.8

These results as well as the criticism by Embden and Schmitz are, however, quite immaterial so far as the method is concerned because it has never been suggested that the method be performed on the urine direct, but only after precipitation by basic lead acetate and ammonia, which wholly removes the effect of the sugar as the following experiments show.

A series of determinations were carried out on a normal urine with and without the addition of 3 grams of glucose per 100 cc. urine. In each instance to 50 cc. urine were added 50 to 75 cc. basic lead acetate solution and 15 cc. concentrated ammonium hydroxide. The mixture was diluted to 500 cc. and 200 cc. of the filtrate, equivalent to 20 cc. urine, were diluted and distilled first with 30 cc. 1 to 1 sulphuric acid (distillate A which was discarded), and then with the gradual addition of potassium bichromate. Some of the distillates were titrated direct and others were redistilled after adding 5 cc. of 10 per cent  $\text{NaOH}$  and 20 cc. of 3 per cent  $\text{H}_2\text{O}_2$ .

cc. $\frac{N}{10}$ iodine used.		
NORMAL URINE NOT REDISTILLED	URINE PLUS 3 PER CENT SUGAR NOT REDISTILLED	URINE PLUS 3 PER CENT SUGAR REDISTILLED
0.4	0.5	0.6
0.6	0.6	0.8
0.6	0.6	0.7
0.6	0.7	

These figures are equivalent to about 30 mgm. of acetone from oxybutyric acid per liter of urine. It is probable that this repre-

<sup>12</sup> The presence of glucose is clearly without effect upon the results when the method is properly carried out. This *Journal*, v, p. 218, 1908.

sents actual oxybutyric acid present in normal urine, but whether or not this is so, the amount is negligible so far as it affects the results obtained from urines containing significant amounts of oxybutyric acid.

When a known amount of oxybutyric acid is added to normal urine, the results by the oxidation method correspond to about 90 per cent of the amount added, as illustrated by the following experiment.

2.540 grams pure *l*-calcium zinc salt were dissolved in 250 cc. normal urine (equivalent to 8.16 grams of the free acid per liter of urine).

	Found grams per liter	10 per cent added to result
Urine without oxybutyric acid.....	0.029	
Urine plus oxybutyric acid.....	7.54	8.29
	7.50	8.25
	7.40	8.14
	7.30	8.03
Average.....		8.18
Amount oxybutyric acid added .....		8.16

There are no essential changes in the procedure from the technique as originally described, but for convenience the description may be repeated here with the addition of some further details.

The oxidation method, combined with the preliminary distillation for the removal and determination of acetone and diacetic acid, is carried out as follows: From 25 cc. to 100 cc. or more of urine (usually 50 cc.) are measured with a pipette into a 500 cc. volumetric flask containing 200 cc. to 300 cc. of water. Basic lead acetate solution (U. S. P.) is added in amount equal to the urine used<sup>13</sup> and the liquid well mixed. Strong ammonia water, about half the volume of the lead acetate, is next poured in, the flask diluted to the mark with water, shaken, and after a few minutes' standing, the liquid is filtered, preferably through a folded filter, 200 cc. of the filtrate is measured into a round bottom flask (800 cc. or liter Kjeldahls are convenient) diluted with water to about 600 cc., 15 cc. of the concentrated sulphuric acid and talc or boiling stone added, and the mixture distilled until about 200 cc. of the distillate have collected (Distillate A).

The distilling flask must be fitted with a dropping tube and water run in from time to time to prevent the volume in the flask from becoming less than 400 to 500 cc.

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<sup>13</sup> If the urine contains but little or no sugar only half the amount or less of lead acetate should be used.

Distillate A, which contains the acetone preformed and from aceto-acetic acid, and which should be collected in a second Kjeldahl flask, is redistilled (for about twenty minutes) after adding 10 cc. of 10 per cent sodium hydroxide.<sup>14</sup> The distillate so obtained ( $A_2$ ) is titrated with standard iodine and thiosulphate solutions.

The residue of urine plus sulphuric acid from which Distillate A was obtained is again distilled<sup>15</sup> dropping in either water, when necessary to keep the volume between 400 and 600 cc., or a dilute solution of potassium bichromate. From 0.5 gram to 1 gram of bichromate will usually be sufficient, and not more than 1 gram should be added unless the liquid turns green indicating a great reduction to chromium sulphate; very rarely 2 or 3 grams of bichromate may be necessary, especially if the sugar has not been completely removed.

A 10 per cent solution of potassium bichromate is kept on hand and 10 cc. of this, diluted to 100 cc. are measured out for each determination. 20 cc. of the dilute solution (0.2 gram  $K_2Cr_2O_7$ ) are first added slowly through the dropping tube and then 10-cc. portions every fifteen or twenty minutes until the whole has been added. Should the liquid become markedly green the bichromate must be added at correspondingly shorter intervals and in amount sufficient to maintain a slight red-yellow color of the chromic acid, which may be detected even in the presence of the green. The distillation is continued with moderate boiling for from two to three hours. The distillate (B), which should be collected in a liter flask to avoid transference, is again distilled for about twenty minutes after adding 10 cc. of 10 per cent sodium hydroxide and 25 cc. of 3 per cent hydrogen peroxide. The flask must be heated cautiously until the peroxide has decomposed. This distillate ( $B_2$ ) is titrated with the standard iodine and thiosulphate.

1 cc. of  $\frac{N}{10}$  iodine = 0.968 mgm. acetone = 1.736 mgm. oxybutyric acid,  
or

1 cc. of  $\frac{1.035N}{10}$  iodine (= 13.13 mgm.  $I_2$ ) = 1 mgm. acetone = 1.793 mgm.  
oxybutyric acid.

#### *Comparison with results by the extraction method.*

The fact that the oxidation method gives results for oxybutyric acid which are uniformly from 5 to 10 per cent too low, explains in part the differences between the results by this method and the values calculated from the levo rotation of the ether extracts,

<sup>14</sup> In many instances, when a high degree of accuracy is not required, this redistillation may be omitted and "distillate A" titrated direct; the results so obtained are slightly higher than those after redistillation from alkali.

<sup>15</sup> The distillation is actually not interrupted; after "A" has collected, a new receiving flask is adjusted and bichromate solution slowly added through the dropping tube. The receiving tube of the condenser must dip below the surface of the water in the receiving flask.

but it appears that this does not explain the differences in all cases. Were this the only factor the results by the oxidation method would regularly be from 90 to 95 per cent of the optical values of the acid extracted by ether, whether the oxidation method were carried out on the urine as usual, or on the solution of the extracted acid. Occasionally this is the case as illustrated by the following figures:

*Diabetic Urine. Grams oxybutyric acid per liter.*

EXTRACTION METHOD (Black <sup>16</sup> )	OXIDATION METHOD	
	On urine	On extracts
4.65	4.75	4.61
5.25	4.73	5.12
5.10	4.80	4.87
5.15	4.77	4.81

But frequently the results from oxidation of the extracts are much below the expected 90 to 95 per cent of their optical value; and after treating the extracts with basic lead acetate and ammonia the oxidation results are still lower. As examples the following may be cited; the results are expressed as grams of oxybutyric acid per liter:

Oxidation method on urine.....	7.71
Black's method (3 hours' extraction).....	7.85
Oxidation of extract.....	6.32=80 per cent
Black's method (4 hours' extraction).....	8.60
Oxidation of extract.....	7.21=84 per cent
Black's method (4 hours' extraction).....	8.76
Oxidation of extract.....	7.58=86 per cent
Oxidation of extract after lead precipitation.....	7.10=81 per cent

The results from solutions 2, 3, 4, 5 and especially solution 10. from normal urine on page 267, show the same point—differences

<sup>16</sup> Black: this *Journal*, v, p. 209, 1908. For the extraction method 50 cc. to 200 cc. of urine were taken and Black's directions followed except that regular Soxhlet extractors were used. The extraction was continued for from six to ten hours, usually in two periods. After removing the ether by cautious warming on the water bath, the residue was dissolved to 25 cc., the solution was shaken cold with a little purified bone black, filtered and polarized. Parts of these solutions were then subjected to determination by the oxidation method, with or without a preliminary precipitation with basic lead acetate and ammonia.

between the results by oxidation and by polarization which are greater than the 5 or 10 per cent already accounted for. As first suggested by Embden and Schmitz<sup>17</sup> there would appear to be present small amounts of a levo-rotary ether-soluble substance which tend to give somewhat too high results by the polariscopic methods. The identity of the substance we do not know. It may be precipitated by basic lead acetate and ammonia, and this preliminary treatment might well be adopted by those who use the extraction method.

There are two other points which we have encountered which tend to low results by Black's technique of the extraction method. Although Black's plan of dehydrating the evaporated urine with plaster and extraction of the dry material is far more rapid and convenient than the Magnus-Levy liquid extraction, the complete extraction frequently requires rather longer than the three or four hours recommended by Black. As a rule we have found in the extract from the second four-hour period 5 or 10 per cent of the amount obtained during the first four hours.

A more serious objection is the occasional apparent decomposition of a part of the oxybutyric acid during extraction. After ten hours and longer extraction we have repeatedly found only about 90 per cent of the acid which we had added to urine or other solutions. Thus from the urine mentioned on page 276, to 250 cc. of which were added 2.540 grams of pure *l*-calcium zinc oxybutyrate (= 8.16 grams oxybutyric acid per liter), duplicate determinations on 100-cc. portions by the Black method (10 hours' extraction) gave 7.675 grams and 7.675 grams per liter.

$$(\alpha = -1.63^\circ, l = 2.2, [\alpha]_D^{20} = -24.12^\circ)$$

Subtracting the blank equivalent to the extract from the urine alone, we have 7.43 grams or only 91 per cent of the amount added. The results of the oxidation method (page 276) represent the expected 90 to 92 per cent of the amount added.

The reason for this loss on extraction is not altogether clear, but is probably due to an oxidation of some of the oxybutyric acid. We have frequently found in the ether extracts a substance which distils off without the addition of any oxidizing agent and which readily reacts in the cold with hypiodate to form iodo-

<sup>17</sup> *Loc. cit.*

form.<sup>18</sup> The substance apparently is not derived from the ether or the plaster, and the preformed acetone and diacetic acid are of course driven off during the preliminary evaporation of the urine. Although the evidence is not conclusive, it is probable that the substance is acetone produced from an oxidation of a little of the oxybutyric acid during the dehydration with plaster or during the extraction.

It appears that Black's application of the extraction method and polarization of the extract usually gives practically correct results, but that the results are somewhat uncertain because they are influenced by the opposing errors of a levo-rotary substance, not oxybutyric acid, tending to give too high values, and on the other hand an occasional incomplete extraction and decomposition of some of the oxybutyric acid, tending to make the results too low. -

The extraction method is very serviceable, although from our experience we prefer for most purposes the oxidation method, because the latter is quicker, requires less manipulation and apparatus, less urine, and especially for small amounts of oxybutyric acid is, with the correction, more accurate.

It is of interest that the parallel determination by the two methods, one of which determines both *d* and *l* forms of the acid, has given no evidence for the occurrence in diabetic urine of *d*-oxybutyric acid. The asymmetric formation of the levo oxybutyric acid, so far as indicated by available evidence, appears to be perfect.

#### SUMMARY.

1. The method for the determination of oxybutyric acid by oxidation to acetone with chromic acid is found to give uniformly about 90 per cent of theoretical values. The results obtained by the method must therefore be corrected by the addition of 10 per cent of the amount found.

2. A procedure for the isolation and purification of oxybutyric acid in the form of a new double salt of calcium and zinc is described.

3. Results by the oxidation method are compared with results obtained by Black's technique of the ether extraction method.

<sup>18</sup> The iodoform-forming substance is usually lost during the removal of the ether on the water bath.



## THE DETERMINATION OF ACETONE.

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In the determination of  $\beta$ -oxybutyric acid through oxidation to acetone by the Shaffer bichromate method<sup>1</sup> with subsequent estimation of the acetone by Messinger's iodimetric titration,<sup>2</sup> difficulty was experienced in obtaining the theoretical amount of acetone from known amounts of pure  $\beta$ -oxybutyric acid. It was suspected that either the Messinger titration was inaccurate or that the acetone was incompletely recovered in the distillates.

Messinger in his original paper,<sup>3</sup> found that in dilute aqueous solutions of acetone, slightly low results were obtained. Collischonn<sup>4</sup> found that the Messinger method gave low results in very dilute acetone solutions. Geelmuyden<sup>5</sup> using the Messinger method obtained satisfactory results on purified acetone in aqueous solutions, but found that, on distillation of such solutions, loss of acetone of from 5 to 10 per cent was unavoidable, even when ice-cooled receivers were used and the solutions were distilled almost to dryness. Denigès<sup>6</sup> claims that the first quarter of the distillate from aqueous acetone solutions contains only about 90 per cent of the acetone present.

In view of the results cited above, it seemed desirable to investigate the accuracy of the Messinger titration and also to determine whether a dilute aqueous acetone solution could be distilled without loss. In addition I have tested the accuracy of the recently described acetone estimation of Scott-Wilson.<sup>7</sup>

<sup>1</sup> Shaffer: this *Journal*, v, p. 211, 1908.

<sup>2</sup> Messinger: *Ber. d. deutsch. chem. Gesellsch.*, xxi, p. 3336, 1888.

<sup>3</sup> *Loc. cit.*

<sup>4</sup> Collischonn: *Zeitschr. f. anal. Chem.*, xxix, p. 562.

<sup>5</sup> Geelmuyden: *Ibid.*, xxxv, p. 503.

<sup>6</sup> Denigès: *Ann. Pharm. de Bordeaux*, 1910.

<sup>7</sup> Scott-Wilson: *Journ. of Physiol.*, xlii, p. 444.

*The Messinger method.*

A sample of acetone prepared from the bisulphite compound (Eimer and Amend) was further purified by distillation over potassium permanganate, and redistillation over fused calcium chloride. The product, which was anhydrous and free from aldehyde, was subjected to fractional distillation and two portions collected between 56° and 57°C.

Dilute aqueous solutions containing known amounts of this purified acetone were prepared as follows. Thin glass bulbs of 2 or 3 cc. capacity, and provided with a capillary side tube, were blown. These were weighed and then filled with acetone,<sup>3</sup> sealed, and again weighed. Each bulb was introduced into a 2-liter glass stoppered volumetric flask nearly filled with water. The bulbs were broken under the surface of the water by a sharp blow from a glass rod; distilled water was then added to the mark and the contents thoroughly mixed.

Bulb I		Bulb II	
Bulb and acetone.....	3.1928	Bulb and acetone.....	2.5639
Bulb empty.....	0.7454	Bulb empty.....	0.9194
	2.4474		1.6745
Corr. for air displacement . .	0.0024	Corr. for air displacement...	0.0017
Acetone.....	2.4498	Acetone.....	1.6762

In measuring out even such dilute solutions as the ones thus prepared, care was necessary in order to obviate loss of acetone. The solution was forced up into the pipette by air pressure from an atomizer bulb, the neck of the flask being closed by a double-holed rubber stopper. In delivering the solution the pipette was always under the surface of water in the receiving vessel. In this manner 25-cc. portions of the solutions prepared as above, were measured into 700-cc. Florence flasks containing each about 500 cc. of distilled water. A measured amount of standardized iodine solution was then run in, 10 cc. of 60 per cent sodium hydrate added, the flasks stoppered, shaken a little, and allowed to stand for five or ten minutes, after which 15 cc. of concentrated hydrochloric acid were added and the liberated iodine titrated with

<sup>3</sup> The bulbs were warmed, and then the tips of side tubes dipped into acetone, so that on cooling the bulbs acetone rushed in.

standard sodium thiosulphate<sup>9</sup> in the usual manner. The following results were obtained.

SOLUTION I.		cc.
Iodine solution added 50 cc.....		(49.8)
Thiosulphate.....		19.0
Iodine used up.....		30.8
Then since 1 cc. of $\frac{N}{10}$ iodine is used up by 0.968 mgm. of acetone		
30.8×0.968×102.8 per cent=30.64 mgm.	} Acetone.	
Present by weighing.....		30.62 mgm.

SOLUTION II.		cc.
Iodine solution added 50 cc.....		(49.8)
Thiosulphate.....		28.6
Iodine used up.....		21.2
Then 21.2×0.968×102.8 per cent=21.09 mgm.		
Present by weighing.....	} Acetone.	20.95 mgm.

The Messinger method, then, is accurate even in quite dilute solutions.

*Distillation of acetone from dilute solutions.*

It is frequently necessary to distil acetone solutions before making the final determinations. I have found, contrary to the results of Geelmuyden and Denigès,<sup>10</sup> that if proper precautions are taken, acetone may be completely distilled off from even a dilute aqueous solution and entirely recovered in the distillate. Ten minutes' distillation is sufficient to accomplish this result, as is shown in the following experiment.

An acetone solution was used, 500 cc. of which when titrated by the Messinger method were found to contain 33.7 mgm. of acetone. This amount of solution was distilled from an 800-cc. Kjeldahl flask, using a block tin condenser connected with a glass delivery tube, the end of which dipped under the surface of about 50 cc. of water contained in the receiving flask. No ice cooling was used or found to be necessary. Distillations were continued for the length of time indicated, and acetone in the distillates immediately determined by the Messinger method.

<sup>9</sup> The thiosulphate was standardized against pure potassium bi-iodate and also against bichromate and found to be 102.8 per cent of  $\frac{N}{10}$ . 50 cc. of the iodine solution were equivalent to 49.8 cc. of thiosulphate, on blank titrations.

<sup>10</sup> *Loc. cit.*

## Determination of Acetone

Time distilled in minutes	Acetone in distillate mgm.
5	31.1
10	33.6
10	33.5
10	33.7
15	33.7
20	33.6
25	33.7
30	33.7

The anomalous results of Geelmuyden and of Denigès may possibly be explained by their failure to always have the end of the delivery tube dip under the surface of the liquid in the receiving flask.

*The mercury cyanide method of Scott-Wilson.*

Although the Messinger method gives correct results and is the most satisfactory for general use when considerable amounts of acetone are to be determined, yet it is not of sufficient delicacy to determine such small amounts of acetone as occur, for example, in a few cubic centimeters of blood.

A more delicate method is that described by Scott-Wilson.<sup>11</sup> This depends upon the precipitation of acetone as a keto-mercury-cyanide compound with subsequent determination of the mercury by titration with a standard sulphocyanate solution under prescribed conditions.

In carrying out the method, as described, several difficulties were encountered, and correct results were not obtained. With certain modifications of the procedure, however, I have found the method to be capable of considerable accuracy with exceedingly small quantities of acetone.<sup>12</sup>

The best results are obtained in the following manner:

Dilute solutions of pure acetone are run into an excess of the recently filtered reagent<sup>13</sup> contained in small Erlenmeyer flasks, allowed to stand

<sup>11</sup> *Loc. cit.*

<sup>12</sup> The method is applicable only for quantities of acetone less than five milligrams.

<sup>13</sup> The reagent is made up as follows: Mercuric cyanide, 10 grams; Sodium hydroxide, 180 grams; Water, 1200 cc. The solution is agitated in a flask and 400 cc. of a 0.7268 per cent solution of silver nitrate slowly run in. At least 30 cc. of the reagent must be taken for each milligram of acetone present.

twenty minutes and then filtered through an asbestos mat<sup>14</sup> in a separable bottom Gooch crucible. By first filtering an aqueous suspension of talcum powder so as to partly close the pores of the filter, less difficulty is experienced in obtaining clear filtrates. In some cases the first portions of the filtrate are turbid and have to be refiltered. The precipitate is washed with cold water until the washings are free from silver.

With the aid of a pointed hooked glass rod the precipitate, mat, and crucible bottom are transferred to a 50-cc. beaker, any adhering particles of the precipitate being washed into the beaker with about 10 cc. of "acid mixture,"<sup>15</sup> 1 cc. of  $\frac{N}{10}$  potassium permanganate is added, the beaker covered with a watch glass, and the liquid boiled until colorless. More permanganate is then added a few drops at a time, until a persistent brown color is obtained which does not disappear on boiling for a couple of minutes. The brown color is then discharged by the addition of a few drops of strong yellow nitric acid. The greater the amount of acetone present the more permanganate is required, and it is essential to the accuracy of the method that an excess be added as indicated above, otherwise the results are low.

The beaker is cooled under the tap, 2 cc. of saturated ferric alum added, and a standard solution of potassium sulphocyanate (approximately 0.1 per cent) run in from a burette until a very faint pinkish brown color is obtained throughout the solution. The end point, which consists in the faintest trace of color, can be detected only when the titration is performed on a pure white surface. A control beaker with one drop excess of sulphocyanate should be at hand for comparison. A whole cubic centimeter of sulphocyanate may be run in after the end point is reached without very greatly darkening the shade.

In the calculation of results Scott-Wilson has assumed that the keto-mercury-cyanide compound has the formula  $\text{HgCOC}_2(\text{HgCN})_4$ , and that consequently 1 mgm. of mercury should be equivalent to 0.058 mgm. of acetone. He determined the value of the sulphocyanate solution in terms of mercury by titrating it against a known mercury solution. In applying this method to the estimation of pure acetone solutions he obtained results about 3 per cent too low. The error he attributed to loss of acetone by evaporation, or to impurities in the acetone. My results which follow, have led me to believe that the error is instead in the method of calculation.

The dilute acetone solution made up from Bulb I, and used as previously described for the Messinger titration was also used in this case. Twenty-five cubic centimeters of this solution were made up to 1 liter with distilled water, and 50 cc. of this latter

<sup>14</sup> Filter paper cannot be used as the strong alkali quickly attacks it.

<sup>15</sup> Nitric acid, 40 parts; Sulphuric acid, 5 parts; Water, 55 parts.

solution, containing 1.53 mgm. acetone, used for each determination. The acetone solutions were each run into 50 cc. of acetone reagent and the estimations carried out as described above.

On titration the following results were obtained, and calculations based on the value of the sulphocyanate solution made as indicated.

KSCN <sup>16</sup>	ACETONE FOUND	ACETONE PRESENT	"ACETONE FACTOR"
cc.	mgm.	mgm.	
23.7 × 0.061.....	1.44	1.53	0.0646
24.1 × 0.061.....	1.47	1.53	0.0635
24.0 × 0.061.....	1.46	1.53	0.0637
23.6 × 0.061.....	1.44	1.53	0.0648

A second acetone solution was made up from the same stock solution from Bulb I, by diluting 25 cc. of this to 250 cc. with water. Of this latter solution 10 cc., containing 1.225 mgm. acetone, were used for each determination.

KSCN	ACETONE FOUND	ACETONE PRESENT	"ACETONE FACTOR"
cc.	mgm.	mgm.	
19.2 × 0.061.....	1.17	1.225	0.0638
18.6 × 0.061.....	1.13	1.225	0.0658

The results are uniformly low. The figures under the heading "acetone factor" represent the value by which each cubic centimeter of potassium sulphocyanate solution used should be multiplied in order to give correct results for the amount of acetone actually in the solution. The average of these values, which is 0.0644, is then to be taken as the true value of the sulphocyanate solution in terms of acetone. From the foregoing it is evident that the sulphocyanate solution cannot be standardized by its mercury equivalent, but that solutions of pure acetone of known strength, as determined by weighing or Messinger titration, can be used to advantage. The discrepancy in the results obtained by using the

<sup>16</sup> The KSCN solution was standardized against a solution of mercuric nitrate, that had been analyzed for mercury by sulphide precipitation. 1 cc. of KSCN was found to be equivalent to 1.05 mgm. mercury, which from Scott-Wilson's formula, would correspond to 0.061 mgm. of acetone.

mercury equivalent as a basis of calculation may be explained by the possibly incorrect formula for the keto-mercury-cyanide compound or by the reaction not being a complete one.

Having thus standardized the sulphocyanate solution, a series of determinations on a different acetone solution was made. The solution used contained 0.172 mgm. of acetone per cubic centimeter, as determined by Messinger titration. Varying amounts of the solution and of acetone reagent were used in order to test the accuracy under different conditions.

ACETONE SOLUTION	ACETONE REAGENT	KSCN	ACETONE FOUND	ACETONE PRESENT
cc.	cc.	cc.	mgm.	mgm.
1	50	$2.65 \times 0.0644$	0.170	0.172
1	50	$2.85 \times 0.0644$	0.183	0.172
5	50	$13.40 \times 0.0644$	0.863	0.860
5	50	$13.50 \times 0.0644$	0.869	0.860
10	100	$27.20 \times 0.0644$	1.75	1.72
10	100	$26.80 \times 0.0644$	1.73	1.72
20	100	$54.00 \times 0.0644$	3.48	3.44
20	100	$54.40 \times 0.0644$	3.50	3.44

The method, then, gives accurate results with varying amounts of acetone and the accuracy is not affected by considerable amounts of acetone reagent in excess of the quantity required. As previously mentioned it is necessary to use at least 30 cc. of the acetone reagent for each milligram of acetone present, or expected to be present.

The acetone reagent is not affected by alcohol, but a precipitate forms with very small amounts of aldehydes, chlorides, hydrogen sulphide, or ammonia. In making determinations, therefore, the absence of these substances must be assured.

If the acetone solution is extremely dilute so that several hundred cubic centimeters are required to make a determination, the results have been found to be somewhat low. In such cases it is necessary to distil the acetone into a smaller volume of water, or better, directly into the acetone reagent. Boiling for ten minutes is sufficient to bring over all of the acetone, and the distillate need not amount to more than 100 cc.

The utilization of this method in the determination of acetone and of  $\beta$ -oxybutyric acid in blood and tissues appears in a subsequent paper.

## SUMMARY.

1. The Messinger method for acetone estimation gives correct results.

2. The Scott-Wilson method gives accurate results only when certain modifications in the original procedure are made. It is applicable to very minute quantities of acetone.

3. In distilling a very dilute acetone solution, all of the acetone may be collected in the distillate within ten minutes.



## NEPHELOMETRIC DETERMINATION OF MINUTE QUANTITIES OF ACETONE.

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(Received for publication, September 4, 1913.)

In order to determine very small amounts of acetone such as occur, for example, in a few cubic centimeters of normal blood, it is necessary to have a method more delicate than those at present in use.

As has been shown in the previous paper, the Scott-Wilson method is a delicate and accurate one for acetone determination, but it is not sufficiently delicate for the small amounts of acetone we wished to determine, so another method was devised.

The addition of acetone to a silver-mercury-cyanide solution gives rise to an abundant white nebulous precipitate. So delicate is the reaction for acetone that 0.01 mgm. is sufficient to cause a distinct opalescence in 50-100 cc. of solution. Further, the density of the opalescence, as measured by the nephelometer, has been found, within limits, to be proportional to the amount of acetone added. The details of the procedure are as follows:

The acetone solution, which must be free from ammonia, aldehyde or hydrogen sulphide, is distilled into an excess of the acetone reagent.<sup>1</sup> The delivery tube must dip under the surface of the

<sup>1</sup> The reagent is made up as follows: Mercuric cyanide, 10 grams; Sodium hydroxide, 180 grams; Water, 1200 cc. The solution is agitated in a flask and 400 cc. of 0.7268 per cent silver nitrate solution slowly run in. Immediately before use the reagent must be filtered through an asbestos mat, the pores of which have been partially occluded by previous filtration of a little talcum in water. At least 30 cc. of the reagent must be taken for each milligram of acetone present or expected to be present. A little experience enables one to tell by the density of the precipitate formed in the first couple of minutes' distillate the approximate amount of acetone present. A dense precipitate may call for the addition of more reagent to the receiving flask.

## 290 Nephelometric Determination of Acetone

liquid in the receiving flask. The distillation is continued for about fifteen minutes or until the distillate measures from 75 cc. to 100 cc. After standing for about half an hour, the distillate is transferred to a graduated cylinder and diluted until an opalescence that can be conveniently read is obtained. The turbidity occasioned by 0.05 mgm. of acetone diluted to 100 cc. is a convenient strength for this purpose, although considerably smaller or larger amounts give good results. With heavy opalescence it is desirable after diluting to a certain volume, say 250 cc., to remove an aliquot portion with a pipette and dilute this appropriately. A solution containing a known amount of acetone<sup>2</sup> is distilled into an excess of reagent<sup>3</sup> and this distillate which is to be used as the standard is diluted as above.

Comparisons of the turbidity of the unknown solutions with that of the standard are made in the nephelometer of Richards.<sup>4</sup>

The nephelometer as originally described may be improved by substituting the telescopic attachment of a Duboscq colorimeter for the eye piece instead of the plain brass tube used by Richards. A further modification consists in a partition between the two tubes. This was designed to eliminate reflections of light from one tube to the other.

Owing perhaps to inaccurate construction of the instrument the same solution when read in both tubes does not necessarily give identical readings. This source of error may be eliminated by making a series of readings, then reversing the tubes and making another series of readings, averaging the two ratios thus obtained; or more simply, as suggested by Kober,<sup>5</sup> by reading the standard solution as an "unknown" and taking this value as the potential height of the standard solution.

As the suspensions slowly settle out, the readings should be made as quickly as possible after filling the tubes.

<sup>2</sup> A convenient stock solution contains about 0.03 mgm. acetone per cc. The strength of such a solution is determined by titration of 200 cc. by the Messinger method.

<sup>3</sup> The solution cannot be added directly to the reagent as a lower result is obtained than when distilled.

<sup>4</sup> Richards: *Zeitschr. f. anorgan. Chem.*, viii, p. 269, 1895; Richards and Wells: *Amer. Chem. Journ.*, xxxi, p. 235, 1904.

<sup>5</sup> Kober: this *Journal*, xiii, p. 485, 1913.

The instrument is manipulated in a dark room, a small electric flash lamp being used to read the scale.

As originally pointed out by Richards<sup>6</sup> the amounts of precipitate are not exactly inversely proportional to the scale readings. Kober<sup>7</sup> has constructed a curve of correction for use with his modification of the nephelometer. When the two solutions for comparison are of nearly the same concentration, the correction is within the limits of observational error and may be disregarded. Further, by using Kober's equation for a correction curve it is seen that the difference between observed and corrected values becomes proportionately less with readings taken with greater depths of solution. If the unknown suspension is so diluted as to be not more than 20 per cent different from the standard and if comparisons are made with scale readings in the neighborhood of 50 mm. or 60 mm., no corrections are necessary.

In doing a series of determinations a single standard suspension is used and the various unknown suspensions are diluted in graduated cylinders to approximately the same opalescence. Little difficulty is experienced in thus obtaining suspensions differing from the standard by not more than 10 per cent.

It is to be mentioned that the nephelometer used was mechanically crude with no vernier and no ratchet and pinion attachments for adjusting the sliding jackets surrounding the tubes. Greater accuracy could possibly be obtained by using a modification of the Duboscq colorimeter. However, quite satisfactory results are possible as is shown below:

Solutions containing varying amounts of acetone were prepared by another member of the staff and determinations made by the writer on these solutions with the following results:

Acetone added	Acetone found
0.015	0.015
0.022	0.021
0.092	0.083
0.28	0.27
0.63	0.65
1.00	0.92
1.54	1.54

<sup>6</sup> *Loc. cit.*

<sup>7</sup> *Loc. cit.*



## THE DETERMINATION OF $\beta$ -OXYBUTYRIC ACID IN BLOOD AND TISSUES.

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(Received for publication, September 4, 1913.)

The Shaffer<sup>1</sup> method for the determination of  $\beta$ -oxybutyric acid is applicable to blood and tissue analysis.

Before applying the method, however, it is necessary to remove proteins and other disturbing substances. Proteins are removed by a modification of the Seegen<sup>2</sup> procedure of sodium acetate precipitation. The paired glucuronic acids, glucose and protein remnants are eliminated by a subsequent precipitation with basic lead acetate and ammonia.

The details of the method when large amounts of defibrinated blood or of tissues are available, are as follows:

A round-bottomed flask of 2 or 3 liters' capacity provided with a dropping funnel is connected with a condenser, the delivery tube of which dips beneath the surface of a little water contained in a 500-cc. receiving flask. The large flask contains 500 cc. of water, 3.5 cc. of glacial acetic acid and a little powdered talc. The liquid is raised to the boiling point. 100 cc. of blood diluted with 400 cc. of distilled water is then run in through the dropping funnel at such a rate that boiling does not cease.<sup>3</sup> Distillation is continued until about 300 cc. have distilled. A very small amount of the liquid may occasionally foam over, but this is of no consequence on account of the subsequent redistillations. This distillate contains preformed acetone plus acetone from diacetic acid. Some

<sup>1</sup> Shaffer: this *Journal*, v, p. 211, 1908; Shaffer and Marriott, *ibid.*, xvi, p. 265, 1913.

<sup>2</sup> Seegen: *Centralbl. f. Physiol.*, vi, p. 604, 1893.

<sup>3</sup> In using hashed organs the hash is all put into the dilute acetic acid before connecting up the apparatus. Care must be taken to shake the flask from time to time to prevent burning at the bottom.

ammonia may be present, hence redistillation with addition of a little dilute sulphuric acid is performed. A second redistillation<sup>4</sup> after the addition of 20 cc. of 3 per cent hydrogen peroxide and a slight excess of alkali serves to destroy or hold back hydrogen sulphide, aldehydes, if any, and volatile acids. The final distillate is used for acetone determination by the Messinger titration in the usual manner.

The large flask is removed from the distilling apparatus, and while the contents are still hot, about 15 cc. of 20 per cent sodium carbonate solution are poured in, with stirring. When sufficient sodium carbonate has been added the dark grumous liquid changes to brown, and a flocculent precipitate settles leaving a clear straw colored supernatant liquid, of amphoteric reaction. The flask is held over a ring burner and the contents boiled for a minute or two, then allowed to cool and transferred to a graduated flask or cylinder and made up to 1000 cc. with water. The whole is thoroughly mixed and filtered through dry paper on a Büchner funnel. An aliquot portion (usually 700 cc.) is transferred to a graduated flask, 30 cc. basic lead acetate solution (U. S. P.) and 15 cc. strong ammonia added and the volume is made up to 1000 cc. The solution is mixed, allowed to stand awhile and filtered on a dry folded filter. 900 cc. of the water-clear filtrate are boiled to expel the greater part of ammonia and to concentrate to about 500 cc. This is cooled and sufficient dilute sulphuric acid added to precipitate the excess of lead present, the lead sulphate is filtered off, 30 cc. of 50 per cent sulphuric acid added and the whole transferred to a liter Kjeldahl flask provided with a dropping funnel. The contents of the flask are distilled and a solution of potassium bichromate or water is run in at such a rate that the liquid always retains some yellow color, and the volume remains between 400 and 500 cc. It is rarely necessary to add more than 0.5 gram of bichromate and an excess is to be avoided. Slow distillation is continued for two hours and 600 to 800 cc. of distillate collected, the precaution being taken that the tip of the delivery tube is always under the surface of water in the receiving flask. The distillate is redistilled with 20 cc. of peroxide and 5 cc. of 10 per cent sodium hydroxide, and the final distillate titrated by the Messinger method.

<sup>4</sup> In a preceding paper it was shown (p. 283) that ten minutes' distillation is ample to distil off all acetone.

To test the accuracy of the method, determinations were made on fresh defibrinated beef blood to which had been added pure synthetic  $\beta$ -oxybutyric acid.<sup>5</sup> The following results were obtained:

100 cc. blood alone: 8.4, 7.9, 8.8 mgm. oxybutyric acid; *average*, 8.3 mgm.

100 cc. blood to which had been added 82.08 mgm. of  $\beta$ -oxybutyric acid as determined on the pure solution: 90.9, 89.6 mgm.  $\beta$ -oxybutyric acid; *average found*, 90.2 mgm.; *amount present*, 90.3 mgm.

Another experiment on a different sample of blood gave the following results:

100 cc. blood alone: 7.2, 7.4 mgm.  $\beta$ -oxybutyric acid; *average*, 7.3 mgm.

100 cc. blood to which had been added 87.1 mgm. oxybutyric acid: 94.3, 92.3, 93.1, 93.9 mgm. oxybutyric acid; *average found*, 93.4 mgm.; *amount present*, 94.4 mgm.

50 grams muscle hash alone, 11.3 mgm.  $\beta$ -oxybutyric acid.

50 grams of same hash to which were added 173 mgm.  $\beta$ -oxybutyric acid: *found*, 182.1 mgm.; *present*, 184.3 mgm.

50 grams liver hash alone gave 16.5 mgm.  $\beta$ -oxybutyric acid.

50 grams of same hash to which were added 173 mgm. of  $\beta$ -oxybutyric acid: *found*, 186.5 mgm.; *present*, 189.5 mgm.

#### *Method for small amounts of blood.*

By determining the acetone in the distillates by the exceedingly delicate method of Scott-Wilson,<sup>6</sup> I have been able to make satisfactory estimations of the acetone bodies in 10-cc. samples of blood, drawn directly from a vein.

The details of the method are as follows:

10 cc. of blood drawn from a superficial vein by a sterile graduated syringe are run into about 40 cc. of 0.5 per cent potassium oxalate solution.

An 800-cc. Kjeldahl flask, provided with a dropping funnel is connected with a condenser, the delivery tube of which dips beneath the surface of water in a receiving flask. The Kjeldahl flask contains 100 cc. of water and 1 cc. of glacial acetic acid. This is brought to a boil and the diluted blood slowly run in through the dropping funnel.

<sup>5</sup> In the form of the purified calcium zinc double salt.

<sup>6</sup> Scott-Wilson: *Journ. of Physiol.*, xlii, p. 444, 1911; Marriott: this *Journal*, preceding paper.

The liquid is kept boiling for about thirty minutes, after the last of the blood has been run in. The distillate is redistilled with a little dilute sulphuric acid and again with 20 cc. peroxide and a slight excess of alkali. The final distillate is caught in small Erlenmeyer flasks containing an excess of the Scott-Wilson "acetone reagent."<sup>7</sup> The delivery tube must dip under the surface of the liquid, and it is not necessary to distil longer than ten minutes in order to get off all of the acetone. The resulting acetone-mercuric-cyanide compound is then filtered off on an asbestos mat in a Gooch crucible and acetone estimated as described in a previous paper.<sup>8</sup> This represents acetone preformed and from diacetic acid.

The residue in the Kjeldahl flask is precipitated while still hot, with about 8 cc. of 10 per cent sodium carbonate, boiled a moment, filtered on a Büchner funnel and washed with hot water. To the clear filtrate are added 15 cc. of basic lead acetate (U. S. P.) and 10 cc. of strong ammonia. This precipitate is allowed to settle and then filtered off on a dry folded filter and the filtrate used for  $\beta$ -oxybutyric acid determination in the same way as described above for large quantities of blood, with the exception that the final distillate is caught in excess of "acetone reagent" and the estimation made by the modified Scott-Wilson method, previously described.

The following results were obtained with freshly drawn dog blood.

Results are expressed in terms of acetone obtained.

*10 cc. of blood alone.*

ACETONE PREFORMED AND FROM DIACETIC ACID		ACETONE FROM $\beta$ -OXYBUTYRIC ACID	
	mgm.		mgm.
	0.03		0.32
	0.03		0.35
	0.03		0.34
Average . . . .	0.03	Average . . . .	0.33

<sup>7</sup> See preceding paper.

<sup>8</sup> *Loc. cit.*



10 cc. of same blood to which had been added 1.74 mgm. acetone as oxybutyric acid.

ACETONE PERFORMED AND FROM DIACETIC ACID	ACETONE FROM OXYBUTYRIC ACID
mgm.	mgm.
0.06	2.12
0.06	2.14
0.06	2.16
Average..... 0.06	Average found... 2.14 Amount present... 2.04

25-gram portions of hashed muscle of a fasting phlorhizinized dog gave the following results:

Messinger method...	{ 7.4 mgm. 7.0 mgm. }	$\beta$ -oxybutyric acid.
Scott-Wilson.....	{ 7.5 mgm. 7.4 mgm. }	$\beta$ -oxybutyric acid.

*Nephelometric method.*

By applying the nephelometer to the determination of the acetone occurring as such or as diacetic acid and also to that obtained from oxidation of the  $\beta$ -oxybutyric acid, it is possible to make a complete analysis using only from 2 to 5 cc. of blood.

For human work, blood is drawn from a superficial arm vein by means of a sterile syringe and run into about 50 cc. of 0.5 per cent potassium oxalate solution, contained in a small weighed flask.

The diluted blood is run into 100 cc. of boiling water acidified with 1 cc. of glacial acetic acid<sup>9</sup> contained in an 800-cc. Kjeldahl distilling flask and the procedure carried out as described on pp. 295-6, with the exception that the amount of the precipitate in the mercury reagent is estimated by means of the nephelometer by the method given on pp. 289-90 in a preceding paper in this number.

The question may arise as to whether the substance giving a precipitate in the acetone reagent is really acetone. In this connection it is interesting to note that the results obtained on blood by my method agree closely with those obtained by the Messinger

<sup>9</sup> Commercial varieties of acetic acid frequently contain substances which behave like acetone. Blank determinations should always be made and correction made accordingly.

iodimetric titration, in which iodoform was produced and identified microscopically. It is true that ammonia, chlorides, aldehydes, and hydrogen sulphide affect the reagent, but the absence of all of these is assured by the procedures adopted.

In view of the fact that the oxidation of oxybutyric acid by chromic acid gives only 90 per cent of the theoretical yield of acetone, as explained in a preceding paper, 10 per cent should be added to the results obtained by titration or by the nephelometer.

A few determinations follow, the results being expressed as milligrams of acetone per 100 grams of blood:

	Acetone plus dia- cetic acid	$\beta$ -Oxybutyric acid
Normal dog.....	0.04	3.2
Normal dog.....	0.08	1.7
Normal dog.....	0.06	1.7
Normal child.....	0.06	4.4
Normal child.....	0.08	4.4
Dog, phlorhizinized.....	7.2	10.4
Child in coma.....	23.4	24.8
Child (following orthopedic operation).....	11.2	28.0

The methods as given above for the estimation of oxybutyric acid have the advantage over the usual optical methods, in that very much smaller quantities of  $\beta$ -oxybutyric acid may be determined with accuracy. The disturbing effect of optically active substances, such as sarcosine, is eliminated. A further advantage in experimental work is that the methods are suitable for estimating optically inactive  $\beta$ -oxybutyric acid. When sufficiently large quantities of oxybutyric acid are present to permit a determination by the optical methods, a comparison of the results with those obtained by the method described in this paper shows the amounts of either dextro, levo, or inactive acid present.

This work was undertaken at the suggestion of Professor Shaffer, and I am greatly indebted to him for his active interest and valuable suggestions.

## STUDIES OF THE ENDOGENOUS METABOLISM OF THE PIG AS MODIFIED BY VARIOUS FACTORS.

### I. THE EFFECTS OF ACID AND BASIC SALTS, AND OF FREE MINERAL ACIDS ON THE ENDOGENOUS NITROGEN METABOLISM.<sup>1</sup>

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(Received for publication, September 10, 1913.)

That there is a fairly constant type of tissue metabolism resulting from the necessary cellular activity which has received the name endogenous metabolism, and which is in great measure independent of the nitrogen intake, is now generally accepted. That the group of metabolic end products of nitrogenous nature present in the urine of an animal whose diet contains no nitrogen shows relationships which are not found in urines under any other conditions is equally well established. It has become the practice, because of lack of knowledge to the contrary, to refer to endogenous metabolism as a single variety; that is, no effort has been made to resolve this type into factors. The most conspicuous and least variable known constituent of the group of endogenous end products of nitrogen metabolism is creatinine. Mendel and Rose<sup>2</sup> studying the conditions under which creatine is eliminated have shown conclusively that in fasting rabbits and dogs, or when the animals are on a diet of fat alone there is always an increase in the output of total creatinine (creatinine plus creatine). This rise is always attended by an increase in the total nitrogen output. They hold that creatinine is derived from creatine, and that those conditions which produce a carbohydrate hunger in the cells of the tissues, lead to excessive catabolism of the tissues and con-

<sup>1</sup> Published with the permission of the Director of the Wisconsin Experiment Station.

<sup>2</sup> This *Journal*, x, p. 247, 1911.

sequent liberation of creatine from the muscles. That further, the animal's power to convert creatine into creatinine, or to destroy it may thereby be exceeded and creatine may appear in the urine. Concomitant with this increased tissue catabolism goes of course an increased elimination of total nitrogen. In harmony with this theory are the data of Myers and Volovic<sup>3</sup> published since the work reported in this paper was carried out. Working with rabbits they found that hyperthermia, whether caused by infection or by keeping the animal in an incubator, leads to an increased elimination of creatinine amounting to 36 per cent over the normal output. The view is expressed that creatinine elimination in fever still represents the normal endogenous metabolism which is here proceeding at an abnormal rate.

It would seem therefore that, if an animal were placed in a condition in which the endogenous type of protein catabolism alone prevailed, and then by some means this type of metabolism could be increased in amount we should anticipate a rise in the creatinine elimination, or an increased creatine output or both. A further question of interest which could be answered by such experiments is the relationship among the constituents of urines carrying nitrogen derived solely from "accelerated endogenous metabolism." Do these relationships always remain the same when exogenous catabolism is absent? We have attempted to throw some light on these questions by employing several methods of varying the intensity in endogenous metabolism in pigs living on a diet of carbohydrate, salts and water. The methods employed in the experiments described in this and in the two papers following, which it was assumed would stimulate protein metabolism, were (1) the addition of hydrochloric acid and acid salt mixtures to the starch diet; (2) the feeding of benzoic acid in the starch diet, and (3) the replacement of starch wholly or partially by fat.

In the present paper we will discuss the effects on endogenous metabolism of giving neutral, basic, and acid salt mixtures, and of free mineral acids with an adequate starch diet. Because of the ease of maintaining an animal during long periods under these experimental conditions we have deemed it best to employ the different methods of accelerating tissue catabolism on the same individuals so that all data would be better correlated and stand-

<sup>3</sup> This *Journal*, xiv, p. 489, 1913.

ardized. For the sake of clearness the data presented in the following tables I to V will be discussed under separate titles.

In view of the exceptional advantages of the pig as an experimental animal it seemed possible to obtain new data concerning endogenous metabolism which can scarcely be gotten with any other type of animal, because it is very easy to place this animal in a condition where the endogenous type of catabolism alone prevails. One of us<sup>1</sup> has called attention to the fact that a pig will eat a diet of starch, water and salts during a long period (36 days) with no sign of disturbance of appetite or loss of weight. Attention was there called to the fact that after a time, which varied somewhat in different individuals, the total nitrogen output in the urine sank to a level where the creatinine N, which remains constant, forms about 18.5 per cent of the total. It was pointed out that this ratio could be used as an index to the maintenance requirements of the animal and would serve as a guide to enable one to feed comparable amounts of any substance to different animals. These observations have now been extended to a considerable number of additional animals and some further comment is necessary on this point. Further experience has shown that some pigs when kept for long periods on the starch, salt and water diet never reach so high a relationship of creatinine N to total N as 18.5 to 100. Others will drop the total N excretion regularly to a point where it is about five and a half times the nitrogen eliminated as creatinine. We have seen animals which even after sixty days on a nitrogen free diet had a ratio of creatinine N to total N of only 10 to 100. Whatever the ratio arrived at, it is very constant. Other pigs will have a ratio of anywhere between 10 and 18.5 to 100. In general it has been the most vigorous and healthy animals which have produced the urines having the highest percentage of creatinine N in proportion to total N. Pigs born in the fall in Wisconsin are frequently chilled so much as to lower their vitality and give them a tendency to pneumonia. It is especially in the fall pigs that we have failed to observe the total N to fall to five and a half times the N as creatinine. The reason for this is not clear. It seems certain to be associated in general with lack of vigor. In a number of instances pigs whose breathing gave evidence of lung affections have persisted in putting out in the urine more total

<sup>1</sup> McCollum: *Amer. Journ. of Physiol.*, xxix, p. 210, 1911.

N than corresponds to the creatinine. Pig No. 34 is an illustration. When given starch and an alkaline salt mixture the N as creatinine was only 9.4 per cent of the total. At autopsy, both lungs were found to be extensively affected with pneumonia.

It seems to be true, however, that the ratios between creatinine N and total N under these experimental conditions is not quite the same for all individuals. Thus in the case of No. 38 during the period on an alkaline salt mixture and starch the per cent of the total N as creatinine was 14.8. At the end of the experiment the animal was killed and examined but microscopically nothing pathological was apparent. If creatinine is a product of the metabolism of muscle tissue alone some variation in the relation between the total end products of tissue catabolism, and creatinine, may be due to variation in the relative sizes of the organs as compared to the total amount of muscle tissue in the body. More experimental data showing such relationships in a considerable number of animals together with a careful study of the composition of the urines will be necessary to throw light on this question.

In an experience with more than thirty pigs it has been found that, the year through, probably three-fourths of all pigs of the sizes employed by us in experimental work, will on a liberal carbohydrate diet come to a stage where the creatinine N will make up about 18.5 per cent of the total, and this ratio can be employed to advantage for the calculation of the lowest level of protein metabolism of which the animal is capable. We have not seen the percentage rise above 18.5 per cent except in a single case formerly reported<sup>5</sup> and this was possibly due to error in the analytical work. Another factor of importance in determining the ratio of creatinine N to total N after a considerable period on the starch diet is the character of the salt mixture supplied. We have repeatedly observed that the lowest level of total-N output is reached only when the salt mixture supplied has an excess of basic over acid radicals. This is due to the fact that when the diet is acid, ammonia is eliminated in quantities sufficient to neutralize the acids present in the diet and this ammonia N is, to a considerable extent, derived from additional protein destruction over what would take place if these acids were not present. This point will be discussed further later on.

<sup>5</sup> McCollum: *Loc. cit.*

In all our experiments agar-agar was given in amounts sufficient to lead to regular evacuation of the intestine so the disturbing factor of the absorption of putrefaction products was kept as low as possible. There is always a regular loss of appreciable amounts of nitrogen derived from the secretions of the tract, and if these residues are not promptly eliminated, some of the nitrogen from this source will be absorbed and eliminated in the urine, changing in some degree the typical relationships between the creatinine N and total N.<sup>6</sup>

EFFECTS OF ACID AND BASIC SALTS AND OF FREE MINERAL ACIDS  
UPON THE ENDOGENOUS NITROGEN METABOLISM.

That neutrality be maintained in the blood and tissues is a fundamental condition of life. It is therefore essential that acid radicals, either ingested or of metabolic origin be neutralized. If acidosis obtains both the fixed cations and ammonia take part in the neutralization. With a normal nitrogen intake the ammonia of the urine under these conditions has been observed to rise while the urea-nitrogen is correspondingly decreased. What would be the result if the metabolism were of the endogenous type alone?

*Experimental.*

The animals employed in these experiments were young pigs brought to their lowest level of nitrogen elimination through several weeks of starch feeding. They were confined in special cages and the urine collected daily according to the methods of McCollum and Steenbock.<sup>7</sup> Upon these samples daily analyses for total N, creatinine N, creatine N, ammonia N and urea N were made. Sulphur determinations were also made during two periods. In the case of the urea N the Benedict-Gephardt method was used rather than the Folin method as a matter of convenience. The small error involved in the use of this method is of no consequence for the purpose of this investigation.

In Table I are found the data for pig 34. The starch diet was begun on January 10 and analyses were commenced with the urine

<sup>6</sup> Compare the papers of Folin and Denis: this *Journal*, xi, p. 167, 1912; Osborne and Mendel: Bulletin of the Carnegie Institution of Washington, No. 156, Part I, p. 39, 1911; Mendel and Fine: this *Journal*, xi, p. 13, 1912.

<sup>7</sup> Research Bulletin No. 21, Wisconsin Experiment Station, 1912.

TABLE I.  
 Pig No. 34: Weight, 31.5 pounds. Starch feeding started Jan. 10.  
*Distribution of nitrogen in urine.*

PERIOD	DATE	VOL. OF URINE		PER CENT OF TOTAL NITROGEN					RATION				
		TOTAL N	UREA N	AMMONIA N	CREATININE N	CREATINE N	REST N	Urea	Ammonia	Creatinine	Creatine	Rest	
I	Feb. 24	1820	1.17	0.69	0.230	0.118	gms.	0.132	59.0	19.7	10.1	11.3	Starch, 60 cal. per kgm.
	25	1980	1.27	0.80	0.250	0.091	gms.	0.129	63.0	19.7	7.2	10.2	NaCl, 5 gms.
	26	1570	1.21	0.72	0.265	0.091	gms.	0.134	59.5	21.9	7.5	11.1	
	27	1360	1.27	0.81	0.178	0.080	gms.	0.202	63.8	14.0	6.4	15.9	
	28	1320	1.56	1.10	0.104	0.093	gms.	0.263	70.6	6.7	6.0	16.8	Starch, 60 cal. per kgm.
II	Mar. 1	1510	1.11	0.80	0.087	0.085	gms.	0.138	72.1	7.8	7.7	12.4	Alkaline salts (I), 12 gms.
	2	1550	1.04	0.69	0.090	0.090	gms.	0.170	66.4	8.7	8.7	16.4	
	3	1530	0.95	0.69	0.080	0.099	gms.	0.081	72.6	8.4	10.4	8.5	
	4	1460	1.09	0.74	0.108	0.084	gms.	0.158	67.9	9.9	7.7	14.5	
	5	1800	1.15	0.81	0.095	0.123	gms.	0.122	70.5	8.3	10.7	10.6	
	6	1600	0.83	0.51	0.076	0.094	gms.	0.150	61.5	9.2	11.3	18.1	
	7	1630	0.92	0.64	0.060	0.091	gms.	0.129	69.6	6.5	9.9	14.0	
	8	1680	0.90	0.52	0.115	0.085	gms.	0.180	57.8	12.8	9.4	20.0	
	9	1700	0.98	0.53	0.098	0.091	gms.	0.261	54.1	10.0	9.3	26.6	
	10	1760	0.90	0.50	0.093	0.080	gms.	0.227	55.6	10.3	8.9	25.2	
	11	1200	0.73	0.39	0.082	0.064	gms.	0.194	53.5	11.2	8.8	20.6	
III	12	1210	1.31	0.85	0.152	0.081	gms.	0.227	64.9	11.6	6.2	17.3	Starch, 60 cal. per kgm.
	13	1530	1.48	0.86	0.322	0.082	gms.	0.216	58.2	21.8	5.5	14.6	Neutral salts (II), 10 gms.
	14	1400	1.28	0.70	0.380	0.078	gms.	0.122	56.9	26.7	6.8	9.0	
	15	1600	1.11	0.89	0.220	0.086	gms.	0.215	56.1	19.8	7.7	10.4	
	16	1400	1.11	0.89	0.220	0.086	gms.	0.215	56.1	19.8	7.7	10.4	





TABLE II.  
Fig No. 38: Weight, 39 pounds. Starch feeding started Feb. 21.  
Distribution of nitrogen in urine.

PERIOD	DATE	VOL. OF URINE cc.	TOTAL N			AMMONIA N			CREATININE N			REST N gms.	PER CENT OF TOTAL N				RATON
			gms.	gms.	gms.	gms.	gms.	gms.	gms.	gms.	gms.		Urea	Ammonia	Creatinine	Rest	
I	Mar. 17	1500	1.94	0.86	0.576	0.166	0.338	44.4	29.7	8.6	17.4	Starch, 90 cal. per kgm. Neutral salts (II), 10 gms.					
	18	1400	2.03	0.75	0.588	0.135	0.557	37.0	29.0	6.7	27.4						
	19	1580	1.53	0.70	0.467	0.131	0.232	45.7	30.5	8.6	15.2						
	20	1800	1.77	0.79	0.562	0.188	0.230	44.6	31.8	10.6	13.0						
	21	1570	1.43	0.67	0.376	0.143	0.241	46.8	26.3	10.0	16.8						
	22	1530	1.58	0.62	0.530	0.188	0.242	39.2	33.5	11.9	15.3						
	23	1640	1.35	0.53	0.406	0.171	0.243	39.3	30.2	12.7	18.0						
	24	1840	1.50	0.57	0.486	0.175	0.269	37.9	32.4	11.7	17.9						
	25	1700	1.45	0.44	0.626	0.159	0.225	30.4	43.1	11.0	15.5						
	26	1520	1.49	0.62	0.425	0.186	0.259	41.6	28.5	12.5	17.4						
	27	1320	1.31	0.55	0.369	0.132	0.259	41.9	28.2	10.1	19.8						
	28	1740	1.53	0.60	0.543	0.186	0.201	39.2	35.5	12.2	13.1						
	29	2140	1.20	0.59	0.394	0.155	0.061	49.1	32.8	12.9	5.1						
II	30	1940	1.01	0.54	0.109	0.191	0.170	53.4	10.8	18.9	16.8	Starch, 90 cal. per kgm. Alkaline salts (IV), 10 gms.					
	31	1470	0.86	0.52	0.071	0.160	0.109	60.4	8.3	18.6	12.7						
	Apr. 1	1740	1.20	0.75	0.070	0.171	0.209	62.5	5.8	14.2	17.4						
	2	1880	1.05	0.66	0.060	0.134	0.196	62.8	5.7	12.8	18.7						
	3	1730	1.24	0.68	0.152	0.185	0.223	54.8	12.3	14.9	18.0						
	4	1950	1.01	0.51	0.156	0.166	0.178	50.4	15.4	10.4	17.6						
	5	1780	1.11	0.63	0.085	0.171	0.212	59.6	7.7	15.4	18.7						
	6	1790	1.14	0.63	0.093	0.162	0.177	59.6	7.5	14.2	18.7						
	7	1930	1.02	0.60	0.098	0.153	0.177	59.6	7.4	14.2	18.7						

	12	1900	1.08		0.158						7.0	10.6	17.4	Starch-fat, 75 cal. per kgm. alkaline salts (III), 10 gms.
III	13	920	1.26	0.82	0.088	0.133			0.219	65.0	6.4	8.5	16.4	
	14	570	1.92	1.32	0.123	0.163			0.314	68.8	6.4	8.5	16.4	
	15	920	1.71	1.09	0.221	0.143			0.256	63.8	12.9	8.4	15.0	
	16	990	1.54	0.95	0.127	0.119			0.344	61.7	8.2	7.7	22.3	
	17	860	1.71	1.14	0.248	0.172			0.150	66.7	14.5	10.1	8.8	
	18	820	1.63	1.03	0.236	0.156			0.208	63.2	14.5	9.6	12.8	
	19	{ urine con-		taminated										
	20													
	21	700	1.39	0.85	0.201	0.129			0.210	61.1	14.4	9.3	15.1	
	22	770	1.57	1.01	0.117	0.192			0.251	64.3	7.5	12.2	16.0	
	23	950	1.05	0.67	0.076	0.121		0.117	0.183	63.8	7.2	11.5	17.4	
	24	1010	0.94	0.54	0.097	0.120		0.130	0.183	57.4	10.3	12.8	19.5	
	25	970	1.32	0.89	0.127	0.146			0.157	67.4	9.6	11.1	11.9	
	26	840	1.32	0.79	0.181	0.133		0.136	0.216	59.8	13.7	10.1	16.4	
	27	710	1.28	0.83	0.102	0.115		0.124	0.233	64.8	8.0	9.0	18.2	
	28	410	1.00	0.62	0.115	0.085		0.095	0.180	62.0	11.5	8.5	18.0	
	29	800	1.91	1.23	0.262	0.174			0.324	61.8	13.2	8.8	16.3	
IV	30	360	1.22	0.73	0.127	0.114		0.105	0.249	59.9	10.4	9.3	20.5	Fat, 55 cal. per kgm. salts (III), 10 gms.
	May 1	330	1.33	0.89	0.129	0.107		0.118	0.204	66.9	9.7	8.0	15.4	
	2	540	1.52	1.04	0.087	0.120		0.137	0.273	68.4	5.7	7.9	18.0	
	3	310	0.94	0.60	0.086	0.100		0.097	0.154	63.8	9.1	10.5	16.4	
	4	370	1.55	1.03	0.190	0.111		0.100	0.219	66.4	12.3	7.2	14.2	
	5	290	1.41	0.83	0.220	0.100		0.101	0.260	58.8	15.6	7.1	18.4	
<i>Summary. Averages by periods.</i>														
I-13 days.....		1.55	0.64	0.488	0.163				0.258	41.3	31.5	10.5	16.7	Starch, neutral salts.
II-14 days.....		1.09	0.65	0.089	0.161				0.183	59.6	8.2	14.8	16.8	Starch, alkaline salts.
III-17 days.....		1.44	0.92	0.155	0.140				0.228	63.9	10.8	9.7	15.9	Starch-fat, alkaline salts.
IV-6 days.....		1.33	0.85	0.140	0.108				0.227	63.8	10.5	8.2	17.0	Fat, alkaline salts.

of February 24. In the first period of four days an ample starch ration with sodium chloride was given. Since no alkaline salts were fed it was necessary for the animal to neutralize the metabolic sulphuric and phosphoric acids by alkali produced within the body. The ammonia elimination is consequently high, being about 19 per cent of the total N excreted, which averaged 1.23 grams per day. (A discussion of the creatinine metabolism will be reserved until later.) There follows a period of eleven days in which a similar starch ration is fed, but with a salt mixture which was of alkaline character (salt mixture I, p. 315). On the first day there was a slight increase in nitrogen elimination, followed immediately by a decrease which continued at the lower level throughout the period. The ammonia N sank to about one-third that of the previous period. It is to be especially noted that there was a distinct drop in the total N. A period of four days followed, in which the alkaline salt mixture was exchanged for an approximately neutral one (salt mixture II). The total N and ammonia N again increased. While the changes are small in themselves they form a large per cent of the total. It is believed that deductions may legitimately be made from such variations, since the experimental error which is of the magnitude 0.01 to 0.02 gram nitrogen, is small compared with the amounts of nitrogen eliminated daily. No exogenous nitrogen was present, and fluctuations have a significance as indicating actual quantitative changes in the metabolic processes.

The experimental work was begun upon pig No. 38 after the animal had received the starch diet for twenty-four days. During the first period of thirteen days a nearly neutral salt mixture was fed (salt mixture II). Under these conditions the ammonia production was high, averaging 0.488 gram, while the total N gave a daily average of 1.55 grams. During the succeeding period of two weeks, the salt content of the ration was changed to one of markedly basic character (salt mixture III). As is shown in Table II the average daily elimination of total N dropped from 1.55 grams to 1.09 grams, the ammonia from 0.488 gram to 0.089 gram, while the urea N and creatinine N remained constant. The very close agreement of the results for urea N in these two periods may be due to coincidence, but there may fairly be deduced from the averages the conclusion that an additional amount of protein has been catabolized in response to the acid character of the diet. One is

impressed with the fact that the organism was apparently not able to utilize the nitrogen of the urea fraction to neutralize the acidity and thus prevent an increased nitrogen elimination. There was no change in the creatinine output and so in accordance with the present conception of protein metabolism the additional protein destruction must have been derived from other sources than muscle tissue.

To demonstrate this point more conclusively one animal, No. 39, was fed 10 cc. of hydrochloric acid (1:4) each day during a period of five days (Table IV). The total N increased from an average of 2.86 grams to 4.03 grams, while the creatinine N gave an average in one period of 0.437 gram and in the other 0.424 gram. The probability that this "extra nitrogen" was derived from some tissues other than muscle is further supported by the observations upon the neutral sulphur in the urine of pig 38 during the different periods (Table III). The neutral sulphur remained con-

TABLE III.

Pig 38.

*Neutral sulphur in urine.*

PERIOD	DATE	VOL. OF URINE	TOTAL N	NEUTRAL S	RATION
		cc.	gms.	gms.	
I	Mar. 19	1580	1.53	0.024	Starch.
	20	1800	1.77	0.022	Neutral salts.
	21	1570	1.43	0.028	
	23	1640	1.35	0.024	
	24	1840	1.50	0.024	
	25	1700	1.45	0.026	
	26	1520	1.49	0.029	
	27	1320	1.31	0.034	
II	Apr. 1	1740	1.20	0.016	Starch, alkaline salts.
	2	1880	1.05	0.019	
	3	1730	1.24	0.021	
	4	1950	1.01	0.023	
	5	1780	1.11	0.030	
	6	1780	1.14	0.033	
	7	1830	1.02	0.035	
<i>Summary. Averages by periods.</i>					
I. 8 days.....			1.48	0.026	
II. 7 days.....			1.11	0.025	

TABLE IV.  
 Pig 39: Weight, 102 pounds. Starch feeding started March 1.  
*Distribution of nitrogen in urine.*

PERIOD	DATE	VOL. OF URINE		PER CENT OF TOTAL N					RATION				
		TOTAL N	UREA N	AMMONIA N	CREATININE N	REST N <sup>a</sup>	Urea	Ammonia		Creatinine	Rest		
		cc.	gms.	gms.	gms.	gms.	gms.	gms.					
I	Apr. 10	3200	2.64	1.33	0.230	0.474	0.606	50.2	8.7	18.0	22.9	Starch, 75 cal. per kgm. Alkaline salts (IV), 25 gms.	
	11	3500	2.75	1.31	0.476	0.472	0.492	47.6	17.3	17.2	17.9		
	12	3340	2.94	1.53	0.374	0.422	0.614	52.1	12.7	14.4	20.9		
	13	2720	2.31	1.33	0.153	0.424	0.403	57.6	6.6	18.4	17.5		
	14	3270	2.54	1.39	0.157	0.482	0.511	54.8	6.2	19.0	20.1		
	15	3000	2.33	1.28	0.144	0.514	0.392	55.0	6.2	22.1	16.8		
	16			urine lost									
	17	3400	2.12	1.17	0.191	0.492	0.267	55.2	9.0	23.2	12.6		
	18	3420	3.26	2.19	0.110	0.595	0.365	67.2	3.4	18.3	11.2		
	19	2970	2.85		0.167	0.548			5.9	19.2			
	20	2780	2.31	1.38	0.111	0.438	0.381	59.8	4.8	19.0	16.5		
	21	2730	2.29	1.42	0.153	0.504	0.213	62.0	6.7	22.0	9.3		
II													Same ration + 4 gms. benzoic acid daily.
	22	2660	2.94	1.51	0.255	0.523	0.652	51.4	8.7	17.8	22.2		
	23	2700	2.57	1.21	0.194	0.476	0.690	47.1	7.6	18.5	26.9		
	24	3020	2.61	1.19	0.193	0.442	0.785	45.6	7.4	17.0	30.0		
	25	2500	2.40	1.24	0.180	0.384	0.596	51.6	7.5	16.0	24.9		

TABLE IV—Continued

III	Apr. 26	3240	5.44	3.03	0.519	0.648	1.243	55.7	9.5	11.9	22.9	Same ration + 10 gms. benzoic acid daily.
	27	3230	2.71	0.85	0.336	0.478	1.046	31.4	12.4	17.7	38.6	
	28	3340	2.46	0.62	0.241	0.460	1.139	25.4	9.8	18.7	45.4	
	29	2580	1.69	0.54	0.124	0.440	0.586	31.9	7.3	23.0	34.6	
	30	3060	2.20	0.48	0.245	0.524	0.951	21.8	11.1	23.8	43.2	
	May 1	2840	2.02	0.47	0.205	0.426	0.919	23.3	10.2	21.1	45.6	
IV	2	2980	2.31	0.52	0.167	0.576	1.047	22.5	7.2	25.0	45.4	Same ration + 16 gms. benzoic acid daily.
	3	2370	2.56	0.49	0.265	0.490	1.315	19.2	10.4	19.2	51.4	
	4	2990	2.87	0.55	0.430	0.478	1.412	19.2	15.0	16.7	49.2	
	5	2940	2.89	0.52	0.402	0.493	1.475	18.0	14.0	17.1	51.0	
	6	3220	3.30	0.69	0.438	0.380	1.792	20.9	13.3	11.5	54.4	
	7	2820	2.69	0.50	0.384	0.342	1.464	18.6	14.3	12.7	54.4	
V	8	3240	3.96	0.57	1.140	0.438	1.812	14.4	28.8	11.1	45.8	Starch, salts (II), 10 gms. HCl (1:4), 10 cc. benzoic acid, 16 gms. daily.
	9	2660	3.96	0.49	1.280	0.404	1.786	12.4	32.4	10.2	45.2	
	10	2450	3.88	0.62	1.220	0.426	1.614	16.0	31.4	11.0	41.6	
	11	2400	4.28	0.43	1.780	0.450	1.620	10.1	41.6	10.5	37.8	
	12	1840	4.09	0.58	1.780	0.404	1.326	14.2	43.5	9.9	32.4	
Summary. Averages by periods.												Starch, alkaline salts. Starch, etc., benzoic acid, 4 gms. Starch, etc., benzoic acid, 10 gms. Starch, etc., benzoic acid, 16 gms. Starch, benzoic acid, HCl, 16 gms.
I-12 days.....			2.56	1.43	0.206	0.488	0.424	55.9	8.0	19.1	16.6	
II- 4 days.....			2.63	1.29	0.206	0.456	0.681	49.1	7.8	17.4	25.9	
III- 7 days.....			2.23	0.58	0.220	0.484	0.948	26.0	9.9	21.7	42.5	
IV- 5 days.....			2.86	0.55	0.384	0.437	1.492	19.2	13.4	15.3	52.1	
V- 5 days.....			4.03	0.54	1.44	0.424	1.632	13.4	35.8	10.5	40.4	

\*This includes hippuric acid N.

stant whether the diet contained a neutral or an alkaline salt mixture.

It might be suggested that the extra nitrogen catabolized with the acid ration had its origin in the liver and that no extra muscle protein was decomposed, which would be in harmony with the constancy of creatinine elimination in the different periods. Hedin<sup>8</sup> has shown that outside the body an acid reaction causes increased proteolysis in liver tissue. Arinkin<sup>9</sup> has obtained similar results. If analogous conditions obtain in the functioning organ the increased nitrogen with constant creatinine elimination would be explained. In this connection it may be recalled that in certain pathological conditions, as in phosphorus poisoning, with its accompanying degeneration of the liver, an increased nitrogen elimination takes place without increased creatinine.<sup>10</sup> Schryver,<sup>11</sup> as a result of his studies upon the autolysis of organs, has advanced the hypothesis that the stability of the liver is the result of the mass action of three sets of bodies, the tissues, the metabolites or bodies derived therefrom, and the autolytic enzymes. An acid reaction causes increased activity of the autolytic enzymes, with the degradation of protein. The resultant amino-acids are the sources of the ammonia necessary to restore neutrality. In cases of starvation or of low nitrogen intake these proteolytic enzymes serve automatically to adjust the destruction of tissues to the requirements of acid neutralization. If this conception be correct we should reason that a nitrogen-free ration of sufficiently alkaline reaction would reduce the endogenous output to a level lower than could be obtained by any other combination of food ingredients. Conversely we should assume that the feeding of a non-oxidizable acid would accelerate the proteolysis. Pathologically, any conditions which would increase the acid formation within the body should also increase the liver autolysis and consequently the amount of nitrogen eliminated. Schryver has cited the examples of insufficient oxygen intake and of phosphorus poisoning. In each instance the diminished oxidation results in the accumulation of intermediate prod-

<sup>8</sup> *Festschrift für Hammarsten*, 1906.

<sup>9</sup> *Zeitschr. f. physiol. Chem.*, liii, p. 192, 1907.

<sup>10</sup> Mendel and Rose: this *Journal*, x, pp. 213-264, 1911, give a full discussion of the literature on this subject.

<sup>11</sup> *Biochem. Journ.*, i, p. 123, 1906.



TABLE 10. Nitrogen excretion and utilization.  
Distribution of nitrogen in the urine.

PERIOD	DATE	VOLUME OF URINE	TOTAL N	UREA N	AMMONIA N	CREATININE N	UNDETERMINED N	PER CENT OF TOTAL N			RATION	
								Urea	Ammonia	Creatinine		
I	May 18	1890	2.30	0.91	0.94	0.226	0.224	39.6	40.8	9.8	9.7	Neutral salts (II), 10 gms.+10 cc. HCl (1 : 4); 75 cal. per kgm. as starch.
	19	1750	1.74	0.72	0.69	0.175	0.155	41.4	39.6	10.1	8.9	
	20	1890	2.03	0.90	0.70	0.234	0.196	44.3	34.5	11.5	9.7	
	21	2080	2.03	0.80	0.73	0.213	0.287	39.4	36.0	10.5	14.1	
	22	2090	1.92	0.77	0.72	0.196	0.234	40.1	37.5	10.2	12.2	
	23	1930	2.27	0.83	0.93	0.236	0.274	36.6	41.0	10.4	12.1	
	24	1730	1.71	0.71	0.62	0.192	0.188	41.5	36.2	11.2	11.0	
	25	2040	1.81	0.75	0.64	0.229	0.191	41.4	35.3	12.7	10.6	
	26	2200	1.86	1.01	0.40	0.211	0.239	54.3	21.5	11.3	12.8	
	27	1920	1.44	0.83	0.25	0.180	0.180	57.6	17.3	12.5	12.5	
II	28	1590	1.68	1.02	0.25	0.245	0.165	60.7	14.9	14.6	9.8	Alkaline salts (IV), 10 gms.; 75 cal. per kgm. as starch.
	29	1480	1.41	0.88	0.19	0.169	0.171	62.4	13.5	12.0	12.1	
	30	2080	1.51	0.93	0.28	0.198	0.102	61.6	18.5	13.1	6.8	
	31	2190	1.44	0.95	0.21	0.202	0.078	66.0	14.6	14.0	5.4	
	June 1	1940	1.78	1.13	0.25	0.214	0.186	63.4	14.0	12.0	10.4	
	2	1700	1.47	0.99	0.19	0.194	0.086	67.3	12.9	13.2	6.5	
	3	1660	1.77	0.85	0.17	0.199	0.551	48.0	9.6	11.2	31.1	
	4	2090	1.92	0.84	0.20	0.230	0.650	43.7	10.4	12.0	33.8	
	5	2180	1.52	0.57	0.16	0.142	0.648	37.5	10.5	9.3	42.6	
	6	2420	1.99	0.70	0.19	0.187	0.913	35.2	9.5	9.4	45.8	
IV	7	1860	1.68	0.42	0.13	0.189	0.941	25.0	7.7	11.3	56.0	Alkaline salts (IV), 10 gms.+10 gms. benzoic acid+2 gms. Na <sub>2</sub> CO <sub>3</sub> ; 75 cal. per kgm. as starch.
	8	2080	1.66	0.35	0.15	0.192	0.968	21.1	9.0	11.6	58.3	
	9	2270	1.65	0.31	0.18	0.188	0.972	18.8	10.9	11.4	58.8	
	10	2310	1.59	0.29	0.15	0.154	0.996	18.2	9.4	9.7	62.6	
Summary. Averages by periods.												
I-7 days.			2.00	0.81	0.76	0.21	0.22	40.5	38.0	10.5	11.0	Neutral salts+HCl.
	II-9 days.		1.60	0.94	0.29	0.21	0.16	58.8	18.1	13.1	10.0	Alkaline salts.
	III-3 days.		1.74	0.75	0.18	0.19	0.62	43.0	10.3	10.9	35.6	Same+5 gms. benzoic acid.
	IV-4 days.		1.65	0.34	0.15	0.18	0.97	20.6	9.1	10.9	58.8	Same+10 gms. benzoic acid.

ucts of combustion (*e.g.*, lactic acid), and it has been demonstrated experimentally that there is also increased nitrogen catabolism. In pathological cases where the liver becomes strongly acid its degeneration is rapid.

The experimental data reported in this paper are in harmony with this hypothesis. The constancy of the creatinine N as contrasted with the marked changes in the total N finds its explanation in the sources of the nitrogen fractions. According to the present theories of protein metabolism, practically all of the creatinine N originates from the muscle tissue, but an acid acceleration of catabolism does not materially affect the creatinine output. It seems probable that the extra nitrogen eliminated under the influence of acid is derived from the liver. If this is the case we should not expect an increase in creatinine output to follow even a marked increase in the total N elimination in acidosis. The experimental data available indicate that the endogenous metabolism of certain tissues can be selectively accelerated by the introduction of acid salts and of hydrochloric acid into the diet.

Two possible explanations seem available for the great excess of nitrogen eliminated by pigs under the influence of acids. It may be assumed that the animal cannot use the nitrogen, which would appear as urea if the diet contained alkaline salts in excess, for the production of ammonia necessary to maintain neutrality in the body. It is also possible that the nitrogen of the urea fraction is utilized in the first instance to produce ammonia and that the nitrogen catabolism of the tissues is stimulated by the presence of the ammonium salts thus formed. Our data do not afford an answer as to the correctness of either of these views. Since these experiments were carried out Underhill<sup>12</sup> has published results which seem to indicate that ammonium salts do stimulate nitrogenous metabolism. There was a large exogenous factor in his experiments and the available data do not seem to warrant a conclusion as to the correctness of the view when the endogenous type of metabolism alone prevails.

<sup>12</sup> Underhill: this *Journal*, xv, p. 327, 1913.

## SUMMARY OF CONCLUSIONS.

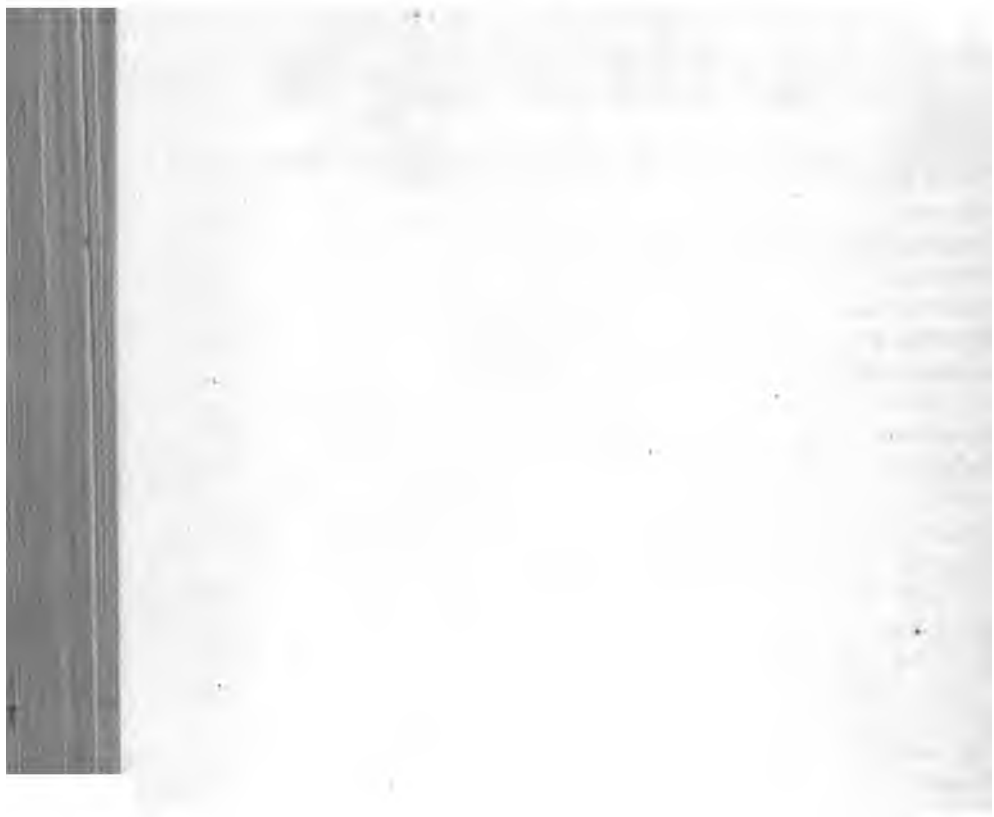
1. Data are presented which show that the endogenous metabolism of the pig reaches its lowest level when the animal has an abundant supply of carbohydrates together with a salt mixture of an alkaline character.

2. The total output of nitrogen derived from endogenous sources can be greatly increased without changing the output of creatinine.

3. The additional nitrogen which is eliminated on an acid over what appears on an alkaline diet is in the form of ammonia. The animal is not able to use the nitrogen of the urea fraction to neutralize the acids present in the diet, but draws additional nitrogen from the tissues for ammonia production.

*Composition of salt mixtures.*

Salt mixture I.		Salt mixture II.	
	<i>per cent</i>		<i>per cent</i>
NaCl.....	0.8	Ca lactate.....	17.4
Ca lactate.....	13.2	MgSO <sub>4</sub> (anhydrous).....	20.7
K <sub>2</sub> HPO <sub>4</sub> .....	22.3	K <sub>2</sub> HPO <sub>4</sub> .....	48.6
CaH <sub>4</sub> (PO <sub>4</sub> ) <sub>2</sub> .....	37.0	NaCl.....	2.8
MgSO <sub>4</sub> (anhydrous).....	2.4	Na <sub>2</sub> SO <sub>4</sub> (anhydrous).....	10.4
Mg citrate.....	17.7	Fe <sub>2</sub> O <sub>3</sub> added in small amount.	
Fe citrate.....	6.6		
Salt mixture III.		Salt mixture IV.	
	<i>per cent</i>		<i>per cent</i>
KCl.....	10.0	Salt mixture I.....	50.0
Ca <sub>2</sub> H <sub>2</sub> (PO <sub>4</sub> ) <sub>2</sub> .....	33.3	K citrate.....	50.0
MgSO <sub>4</sub> (anhydrous).....	6.7		
Na <sub>2</sub> CO <sub>3</sub> (anhydrous).....	50.0		
Fe <sub>2</sub> O <sub>3</sub> added.			



## STUDIES OF THE ENDOGENOUS METABOLISM OF THE PIG AS MODIFIED BY VARIOUS FACTORS.

### II. THE INFLUENCE OF FAT FEEDING ON ENDOGENOUS NITROGEN METABOLISM.<sup>1</sup>

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(Received for publication, September 10, 1913.)

Many investigators have called attention to the fact that feeding fat alone does not spare protein catabolism in the same degree as do carbohydrates.<sup>2</sup> In fact Cathcart and Landergren have observed that in dogs fat feeding tends to increase nitrogen elimination in a marked degree. The recorded experiments of this character are all of short duration, and no accurate standardization of the lowest possible level of protein metabolism of the experimental animals was made. We deemed it of interest to test the question of the influence of a liberal supply of calories as fat on the endogenous metabolism of pigs which had been carefully reduced to their lowest level of nitrogen output by long continued feeding of a diet of starch, salts and water. The preliminary record would in such experiments serve as a standard for total nitrogen and creatinine elimination, with which the values found under the influence of fat feeding could be compared.

It is difficult to feed a sufficient energy intake in the form of pure fat. Our first attempts, using lard, were not very successful, but butter fat, being much more palatable is taken much more readily by pigs. Since the amounts of fat required in these

<sup>1</sup> Published with the permission of the Director of the Wisconsin Experiment Station.

<sup>2</sup> Voit: *Handbuch der Physiologie*, Leipzig, 1881, Vol. VI, Part I; Landergren: *Skand. Arch. f. Physiol.*, xiv, p. 112, 1903; Cathcart: *Journ. of Physiol.*, xxxix, p. 311, 1909; Siven: *Skand. Arch. f. Physiol.*, x, p. 91, 1900; xi, p. 303, 1901.

experiments tend to produce diarrhea we gave the animals paper pulp made from filter paper, with the fat. Salts were given to No. 34 in a mixture nearly neutral (salt mixture II, page 315) and to No. 38 in the form of a decidedly basic mixture (salt mixture III, page 315).

In the case of pig No. 34 (Table I, page 304), 80 grams of butter fat, without any starch, were consumed in the periods following March 25, together with a neutral salt mixture. On the first day there was a marked rise in the nitrogen output, but the average for the period is practically the same as in the preceding starch periods where the same salt mixture was taken. This rise was not accompanied by an increased output of creatinine. During the last period the animal became weakened and there was a decided drop in the output of creatinine.

During the periods of fat feeding with this pig creatine appeared in the urine, and in a few days exceeded in quantity the creatinine nitrogen. Creatine was found in the urine of a number of days in the period when both carbohydrates and fat were fed. The appearance of creatine during exclusive fat feeding is in agreement with the observations made upon other species.<sup>3</sup> Acetone and diacetic acid were found only during the last few days of fat feeding.

Pig No. 38 (Table II, page 306) refused on a number of days to eat the full quota of fat. The complication of a deficient energy intake therefore arises, but it is probable that the energy requirement was nearly covered during the entire time. Attention is, however, called to the fact that there was *no sustained rise in the nitrogen output under the influence of butter-fat feeding*. In this connection it might be urged that it is possible that the output of total nitrogen resulting from endogenous metabolism may have fallen to a decidedly lower level than it was in the earlier periods of the experiment. The marked fall in the output of creatinine during the final period (IV) would lend some support to this assumption. If this were the case, the maintenance of the total-N output, under the influence of fat feeding, at nearly the same level as in the earlier periods on starch would in reality be an acceleration of the endogenous metabolism, the total output of nitrogen

<sup>3</sup> Mendel and Rose: this *Journal*, x, pp. 213-264, 1911; Myers and Fine: this *Journal*, xv, p. 305, 1913.

being increased without any corresponding increase in the creatinine output. The constancy of the rest nitrogen, and the uniformity in the amounts of ammonia in the third and fourth periods point strongly against such an assumption, and we are of the opinion that there was no sustained stimulation of tissue catabolism as the result of the pure fat diet. It is also of great interest that if creatine was present at all in the urine of No. 38 it was in *very small amounts*, while in the urine of No. 34 large amounts of creatine were present on the same diet. The only difference in the diets of the two pigs was in the character of the salt mixture supplied. With the basic salt mixture creatine was present in *small* amounts or entirely absent, while with the neutral salts the amount was large. We call attention to this point only incidentally at this time since we are making an extended study of the creatine metabolism of the pig in this laboratory. The data available do not warrant conclusions, but the above observation is of interest in connection with the many observations on the conditions under which creatine is eliminated by other animals. The feeding of protein is attended with the production of metabolic acids derived from the sulphur and phosphorus, and of fat by a tendency toward the accumulation of organic acids arising as intermediate products of oxidation. It is possible that this factor is of importance in causing an elimination of creatine.

McCollum and Steenbock<sup>4</sup> have called attention to the fact that moderate fasting does not lead to the elimination of creatine in the pig as it does in other species and attribute this difference to a greater ability of the pig to utilize fat for energy production. This is supported by the observation that even with high fat feeding acetone and diacetic acid are not eliminated except after a considerable time and then in small amounts only. This idea is also further supported by our further observation that the feeding of butter fat does not cause a permanent rise in the excretion of nitrogen in pigs reduced to their endogenous level through long continued carbohydrate feeding.

<sup>4</sup> McCollum and Steenbock: this *Journal*, xiii, p. 209, 1912.

## SUMMARY OF CONCLUSIONS.

1. Feeding fat as the sole source of energy does not lead to a sustained rise in the nitrogen output of pigs which have been reduced to their lowest possible level of nitrogen metabolism by long continued starch feeding.

2. Fat feeding may produce a considerable elimination of creatine. The total creatinine (creatinine+creatine) may be greatly increased without a corresponding rise in the total nitrogen output.

3. The possibility of the acid or basic character of the ration having an influence on the creatine production is suggested.



## STUDIES OF THE ENDOGENOUS METABOLISM OF THE PIG AS MODIFIED BY VARIOUS FACTORS.

### III. THE INFLUENCE OF BENZOIC ACID ON THE ENDOGENOUS NITROGEN METABOLISM.<sup>1</sup>

By E. V. McCOLLUM AND D. R. HOAGLAND.

(From the Laboratory of Agricultural Chemistry of the University of Wisconsin.)

(Received for publication, September 10, 1913.)

The experimental feeding of benzoic acid and benzoates to various animals has been reported by a great number of investigators.<sup>2</sup> The object of many of these investigations has been to determine whether glycocoll can originate *de novo* in the body for hippuric acid synthesis, or whether hippuric acid can be produced under the influence of benzoic acid or benzoate feeding only in so far as the proteins of the food or the body tissues decompose and yield the glycocoll complex. The conclusions reached as the result of these studies have been various. The more recent papers of Ringer<sup>3</sup> and of Epstein and Bookman<sup>4</sup> summarize the views that have been expressed on this point.

No results have been reported which involve the use of animals reduced to their minimum level of nitrogen output while receiving nitrogen-free food of ample calorific value. A vigorous pig, eating a liberal ration of starch, salts and water, will readily take relatively large doses of benzoic acid for a long period without loss of appetite or signs of illness. It is thus possible to attain complete control of experiments in which the endogenous type of metabolism alone prevails and to obtain ample amounts of urine for the estimation

<sup>1</sup> Published with the permission of the Director of the Wisconsin Experiment Station.

<sup>2</sup> See Wiechowski: *Beitr. z. chem. Physiol.*, vii, p. 204, 1905-06. All the older literature on hippuric-acid synthesis is referred to and discussed here.

<sup>3</sup> Ringer: this *Journal*, x, p. 328, 1911.

<sup>4</sup> Epstein and Bookman: this *Journal*, x, p. 353, 1911.

of all the determinable nitrogen compounds eliminated, and at the same time to continue the experiments long enough to make the results conclusive. Relatively long preliminary periods on a starch diet serve to standardize the experimental animal in a manner more satisfactory than can be attained with any species which has been employed hitherto.

Although no complete resumé of the literature of the subject is desirable here it may be well to refer to a few of the experiments most closely related to our own, which will serve to show the lack of unanimity of opinions arrived at by others. Parker and Lusk,<sup>5</sup> employing rabbits, reached the conclusion that only 3-5 per cent of the total nitrogen of starvation can appear as hippuric acid when benzoic acid is fed, which represents an amount of glycocholl which would be yielded by the proteins of the body on acid hydrolysis. Wiechowski,<sup>6</sup> employing guinea pigs, found that moderate doses of benzoic acid do not lead to an increased nitrogen elimination and observed the ratio of hippuric acid N to total N to be as high as 64 : 100. He believes that glycocholl can be synthesized at the expense of urea. Magnus-Levy<sup>7</sup> criticized the method of calculation of Wiechowski, and holds that his values are excessive, but is essentially in accord with the latter in regarding the glycocholl content of the metabolized tissues insufficient to account for the hippuric acid eliminated.

Ringer<sup>8</sup> has recently employed both rabbits and goats and concluded that the ingestion of benzoic acid caused an increased nitrogen elimination, and that from the nitrogen of this "extra destroyed protein," is derived the glycocholl for hippuric acid synthesis. Urea nitrogen he found not materially affected, but observed as high as 36 per cent of the total nitrogen appearing as hippuric acid. Ringer holds that the glycocholl resulted not from a deviation of the course of normal intermediary metabolism, but had its origin rather in a specific and peculiar metabolic process.

Epstein and Bookman,<sup>9</sup> employing rabbits, concluded that benzoic acid exerts a truly toxic effect, but acts in a selective way, causing the elimination of excessive amounts of nitrogen which is almost entirely accounted for in the hippuric acid eliminated. It is apparent therefore that a clarification of existing views is highly desirable.

We were led to believe from the recorded data, that the ingestion of benzoic acid would stimulate endogenous metabolism, when this type of protein catabolism alone prevailed. That this is not the

<sup>5</sup> Parker and Lusk: *Amer. Journ. of Physiol.*, iii, p. 472, 1900.

<sup>6</sup> Wiechowski: *loc. cit.*

<sup>7</sup> Magnus-Levy: *Biochem. Zeitschr.*, vi, p. 521, 1907.

<sup>8</sup> Ringer: *loc. cit.*

<sup>9</sup> Epstein and Bookman: *loc. cit.*

case is made apparent by an inspection of Tables IV and V, pages 310 and 313. The experimental procedure was the same as that employed in the experiments described in the two preceding papers. The pigs were reduced to their lowest level of nitrogen elimination by a preliminary starch period, so that the factors of energy intake and exogenous protein metabolism are eliminated. The acid or basic character of the ration has been varied under controlled conditions. The experiments are believed to be of sufficient duration to give reliable data concerning the points under consideration.

During the first period of twelve days pig No. 39 (Table IV, p. 310) received a ration of starch plus alkaline salts (salt mixture IV). Having obtained the necessary preliminary data, 4 grams of benzoic acid per day were added to the ration, in two feeds. No marked rise in the total nitrogen output was observed. The urea N decreased, while the ammonia N remained constant.

On April 26 the daily dose of benzoic acid was increased to 10 grams. There occurred a sudden rise in all the nitrogenous constituents of the urine. The total N rose to more than double that of the preceding day. The creatinine, though increased, was not in proportion to the total. On the following day the total nitrogen sank again, and the average for the rest of the period is slightly less than for the preliminary period. Of special interest is the average daily elimination of urea N. This decreased to less than one-half the quantity excreted in the previous periods. The decrease is in fact great enough to account for all the nitrogen necessary for the synthesis of hippuric acid equivalent to the benzoic acid fed. During the following period the dose of benzoic acid was increased to 16 grams per day. A rise in the total N output followed, while the urea N remained practically constant at its minimum level.

In the final period the salt mixture was changed from an alkaline to a neutral one and 10 cc. of 1 : 4 HCl were added in addition to the benzoic acid, as a part of the work discussed in the first paper of this series (p. 299). It was thought of interest to observe the effect of superimposing upon the endogenous metabolism, a demand for nitrogen, both for neutralization of the acid, and for hippuric acid synthesis. The result was a marked rise in total-N and ammonia-N output. The previous low figure for urea N remained practically unaltered.

The work with pig No. 39 was duplicated with pig No. 43 and the data are presented in Table V (page 313). The data obtained with this pig confirm those of the former animal in all respects. Under the influence of benzoic acid the total nitrogen output was not increased unless the dose was very large, and then the rise was only temporary. The urea nitrogen drops at once and hippuric acid is without doubt produced from the nitrogen which would have appeared in this fraction had no benzoic acid been given. The ammonia nitrogen is not changed in amount by the presence of benzoic acid in the diet. In the case of pig No. 43, weighing only 49 pounds, the daily dose of 10 grams of benzoic acid is relatively high and it seems improbable that larger amounts would lead to data of greater significance than are afforded by these experiments. In this table as in table IV it is apparent that the temporary rise in the nitrogen output at the beginning of the benzoic acid feeding affects the creatinine output but little if at all.

From a consideration of these results it seems fair to conclude that in the body of the pig, a considerable portion of the nitrogen which, in the normal metabolic processes would be converted into urea, may be diverted to the synthesis of hippuric acid. Excessive doses of benzoic acid will not, however, serve to reduce the urea-N fraction below a certain constant level, viz., about 20 per cent of the total. An increase in the total nitrogen output will occur instead. This view is essentially in harmony with that of Wiechowski, and is at variance with those of Ringer and of Epstein and Bookman. It seems probable that the discrepancies may find an explanation in the very short period of observation made by these investigators. In experiments of very short duration, complicated by the presence of exogenous protein catabolism, excessive doses of benzoic acid, causing a sudden increase in the output of total nitrogen may entirely obscure the relation between hippuric acid and urea. It is of course possible that different species of animals may react differently under these experimental conditions.

SUMMARY OF CONCLUSIONS.

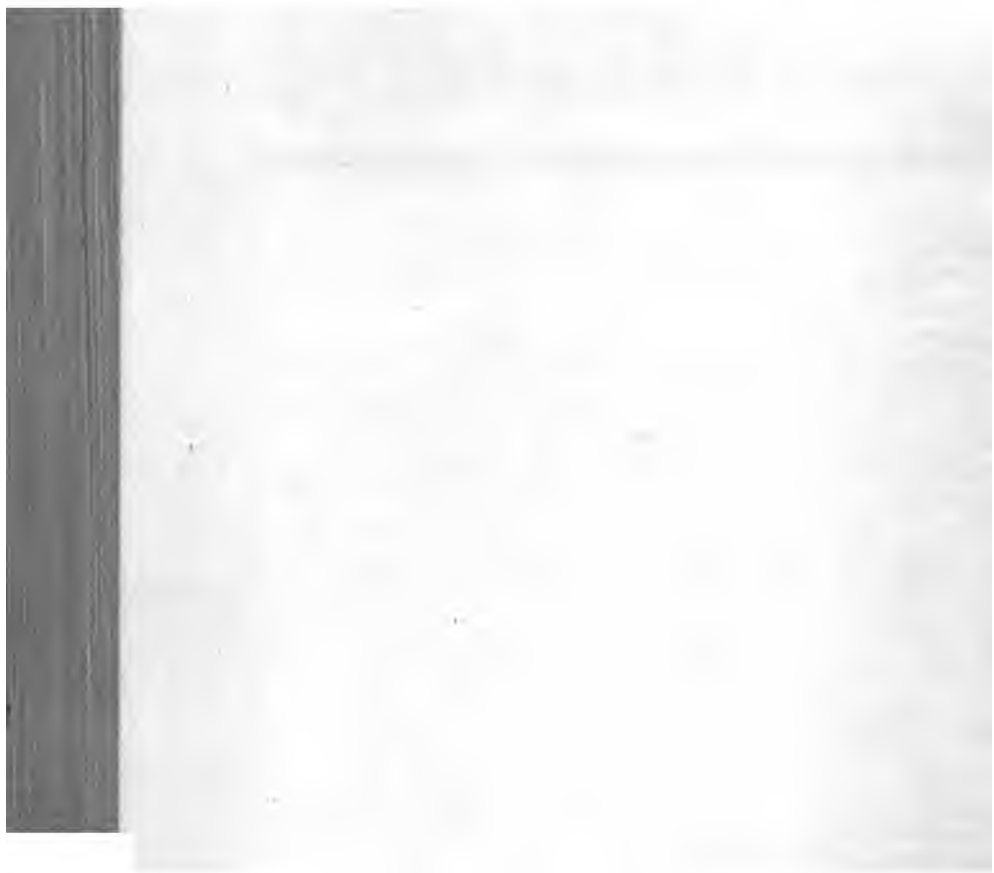
1. A considerable amount of the nitrogen which appears in the form of urea in pigs reduced to the endogenous level of protein metabolism, may be converted into glycocoll when benzoic acid is fed, for the purpose of hippuric acid synthesis.

2. When the quantity of benzoic acid ingested is not excessive, there is no noticeable rise in the total nitrogen excreted, over that which is eliminated on the same diet without benzoic acid.

3. When the quantity of benzoic acid ingested is very large, there is a marked increase in the output of total nitrogen catabolized. The urea nitrogen cannot be reduced to a lower level than about 20 per cent of the total.

4. No change in the creatinine output is observed when the protein catabolism is stimulated by excessive doses of benzoic acid.

5. Endogenous protein metabolism appears to consist of at least two types. One can be stimulated greatly for ammonia production by the introduction of mineral acids, or for hippuric acid when benzoic acid is introduced; the other, measured by creatinine, remains unaffected by the methods we have described.



## THE NON-INTERFERENCE OF "PTOMAINES" WITH CERTAIN TESTS FOR MORPHINE.

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(Received for publication, September 26, 1913.)

In trials for murder by morphine poisoning the defense often insists that the reactions obtained have been, or may have been, caused by ptomaines and not by morphine. It has often been stated that many tests for alkaloidal poisons may be simulated so closely by various bacterial products formed during the putrefaction of biological material that it becomes impossible to distinguish between them.<sup>1</sup>

Vaughan<sup>2</sup> obtained by anaerobic putrefaction of certain tissues, a substance which he thought gave certain reactions similar to those due to morphine and claimed "*that the tests for morphine by following the scheme of Dragendorff are altogether untrustworthy.*" Witthaus,<sup>3</sup> however, claims in contradiction to Vaughan *that if a residue obtained by a properly conducted Dragendorff or other appropriate method gives the following reactions, ferric chloride, Fröhde's, Pellagri's, Husemann's, nitric acid, and iodic acid, with distinctness and purity, the proof of the presence of morphine is quite as complete as it is in the case of nitric acid, whose presence is unhesitatingly assented by chemists upon the evidence of color reactions.*

We would like to state that on starting the work to be described in this paper we felt that Vaughan's idea was correct, and this

<sup>1</sup> It has been claimed that bacterial products may give reactions similar to those due to morphine, conine, nicotine, atropine, strychnine, digitalin, veratrine, colchicine, and delphinine.

<sup>2</sup> *Trans. Assoc. Amer. Phys.*, ix, p. 249, 1894; Peterson and Haines: *Text-book of Legal Med. and Toxicology*, 1904, 2, p. 690.

<sup>3</sup> Witthaus and Becker: *Med. Jurisprudence, Forensic Med. and Toxicology*, 1911, iv, p. 999.

work was commenced with the hope of being able to devise some method to distinguish the reactions of morphine from putrefactive products. However, we found no difficulty in distinguishing the reactions due to morphine from those due to putrefactive products and agree therefore with the claim of Witthaus stated above.

*Methods.* About 5 kgm. of a mixture of human liver, pancreas, kidney, intestines, stomach, brain and heart muscle were chopped finely and divided into two portions. One portion was placed in a wide mouthed jar, exposed to the air and allowed to putrefy for fifty days (Solution A). The other portion was placed in a large bottle and securely stoppered with a perforated cork connected with a bent glass tube. The cork was sealed with paraffin and the outer end of the glass tube was allowed to dip into a cistern of mercury thus excluding all communication with the outside air. This tissue in the bottle was allowed to stand for fifty days (Solution B). At the end of this time the contents of the two bottles were poured into separate dishes.

It may be seen that the bottle "Solution A" contained the products of aerobic putrefaction of certain human organs while "Solution B" contained the products of anaerobic putrefaction of the same organs. Solutions A and B were then divided into four portions and to two portions of each solution 150 mgm. of morphine sulphate were added. One portion of Solution A (aerobic putrefaction) without addition of morphine sulphate and one portion with addition of morphine sulphate were then subjected to the Stas-Otto method of extraction. Similar portions of Solution A were also subjected to the Dragendorff process. One portion of Solution B (anaerobic putrefaction) without addition of morphine sulphate and one portion with addition of morphine sulphate were subjected to the Stas-Otto method. Similar portions of Solution B were also subjected to the Dragendorff process.<sup>4</sup>

The following tabulated summary contains the results obtained in this study, *showing that bacterial products formed during aerobic and anaerobic putrefaction of certain human organs did not in any way give reactions simulating those due to the presence of morphine*

<sup>4</sup> The Stas-Otto method was carried out as described by Autenrieth: *The Detection of Poisons and Strong Drugs*, 1909; the Dragendorff method was carried out as described by Witthaus (*loc. cit.*, p. 157).



and in no way interfered with the detection of morphine, when morphine was added to these putrefactive products.

In the following tables + means that a typical reaction was obtained, while - means that no color at all was produced by the reagent. In those cases where the color is mentioned, it indicates that while the reaction was negative still a color was produced by the reagent. The various tests mentioned for morphine were carried out according to the directions given by Witthaus.

TABLE I.  
Results of Stas-Otto method of extraction.

EXTRACTS	No morphine sulphate added to these solutions.														
	SOLUTION A (AEROBIC)							SOLUTION B (ANAEROBIC)							
	TESTS							TESTS							
	Nitric Acid	Husemann's Pellagris'	Fröhde's	Marquis'	Iodic acid	Ferric chloride	Prussian blue	Nitric acid	Husemann's Pellagris'	Fröhde's	Marquis,	Iodic acid	Ferric chloride	Prussian blue	
ner extract of acid olution.....	pale pink	-	dirty brown	-	-	dirty gray	+	pale pink	-	-	-	-	gray	+	
her extract of odium hydroxide olution.....	-	-	pink	-	-	-	+	-	-	-	-	-	-	+	
ner extract of am- moniacal solution.	-	-	dirty brown	-	-	-	+	-	-	-	-	-	-	+	
loroform extract of ammoniacal sol.	-	-	-	-	very pale pink	-	+	-	-	-	-	pale pink	-	+	
150 mgm. of morphine sulphate added								150 mgm. of morphine sulphate added							
ner extract of acid olution.....	pale pink	-	brown	-	-	-	+	pale pink	-	-	-	-	-	+	
her extract of odium hydroxide olution.....	-	-	brown	-	-	-	+	-	-	-	-	-	-	+	
her extract of am- moniacal solution	+	++	+	+	+	?	+	+	++	++	+	+	?	+	
loroform extract of ammoniacal sol.	+	++	+	+	+	+	+	+	++	++	+	+	+	+	

## Tests for Morphine

TABLE II.  
Results of Dragendorff extraction.\*

EXTRACTS	No morphine sulphate added to these solutions														
	SOLUTION A (AEROBIC)							SOLUTION B (ANAEROBIC)							
	TESTS							TESTS							
	Nitric acid	Husemann's	Pellagris'	Fröhde's	Marquis'	Iodic acid	Ferric chloride	Prussian blue	Nitric acid	Husemann's	Pellagris'	Fröhde's	Marquis'	Iodic acid	Ferric chloride
Petroleum ether ext. of acid sol.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Benzene extract of acid solution	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Chloroform ext. of acid sol.	pink	-	-	-	-	-	+	pink	-	-	-	-	-	-	+
Ether extract of acid sol.	pink	-	-	-	-	-	+	pink	-	-	-	-	-	-	+
Petrol. ether ext. of ammon. sol.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Benzene extract of ammoniacal sol.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Chloroform ext. of ammoniacal sol.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Amyl alcohol ext. of ammon. sol.	-	-	-	-	-	-	-	brown yellow	-	pink	-	-	-	violet	green
150 mgm. of morphine sulphate added								150 mgm. of morphine sulphate added							
Petroleum ether ext. of acid sol.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Benzene extract of acid solution	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Chloroform ext. of acid solution	pink	-	-	-	-	-	+	-	-	-	-	-	-	-	+
Ether extract of acid solution	pink	-	-	-	-	-	+	-	-	-	-	-	-	-	+
Petrol. ether ext. of ammon. sol.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Benzene ext. of ammon. sol.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Chloroform ext. of ammoniacal sol.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Amyl alcohol ext. of ammon. sol.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

\* These results also show the absolute worthlessness of the Prussian blue test.

## BACTERIAL AND ENZYMIC CHANGES IN MILK AND CREAM AT 0°C.<sup>1</sup>

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### INTRODUCTION.

Previous work<sup>2</sup> in this laboratory has demonstrated that raw milk, held at or a little below a temperature of 0°C., undergoes a marked proteolysis, which is pronounced at the end of two weeks. One function of the present research was to determine what part of this proteolysis must be ascribed to the native enzymes of the milk and what part to the bacterial flora, and, finally, to determine the combined action of these two agents. The action of the milk

<sup>1</sup> This paper was presented at the Third International Congress of Refrigeration, Chicago, 1913. It is the report of work done during the winter of 1908-09, when the laboratory was confronted with the task of studying the effect of low temperatures on flesh foods. There were no precedents or methods to guide us, and the apprehensions of the public demanded that results be obtained promptly lest the public health suffer. We, therefore, made a series of observations on the chemical and bacteriological changes occurring in milk and cream, to determine as quickly as possible the general trend of the decomposition at low temperatures, and conducted our work on flesh changes accordingly. Part of the preliminary work has already been published in this *Journal*; the remainder constitutes the present communication, issued in the hope that the fundamental facts which developed as the work progressed, may be of service to others as they were to us. It would have been impossible to execute such a study promptly had there not been unusually efficient team work on the part of the investigators, and an intelligent cross interest of chemists and bacteriologists in the entire scope of the work. Dr. J. S. Hepburn, in addition to his share of the chemical analyses, has correlated and presented the data, and to him the thanks of all the authors are due.—M. E. PENNINGTON.

<sup>2</sup> This *Journal*, iv, p. 353, 1908.

enzymes took place in formolized raw milk, of the bacterial flora in reinfected sterile milk, and of the two agents in combination in raw untreated milk. Sterile milk was also studied as a control.

Since in the previous studies, it was shown that the acidity of raw milk increases<sup>3</sup> and that the lactose content decreases,<sup>4</sup> when raw milk is held at 0°C., it was considered advisable to include within the scope of the present investigation, determinations of acidity and lactose, and to line up the chemical changes with the changes in the freezing point. At the same time, a similar set of experiments was carried out on cream, the chemical analysis including the various fat constants, the lecithin and the freezing point. Certain zymochemical investigations were made on both the milk and the cream. The research cited showed that bacterial growth to a high degree had taken place in the milk during its progress,<sup>5</sup> so the number, groups and species of organisms, present in the various samples of milk and cream, were determined. The bacterial work possessed an added interest, inasmuch as it rendered possible a comparison of the relative rate of growth of the milk organisms in reinfected sterilized clean milk (or cream) and raw untreated clean milk (or cream) at a temperature of 0°C.

#### METHODS.

##### *Preparation of the samples.*

The raw milk was fresh clean milk from a high-grade dairy and was strictly comparable with the milk certified by medical milk commissions. The portion for the study of sterile and reinfected sterile milk was received at the laboratory two days in advance of the remainder of the sample, and was sterilized by heating in an Arnold steam sterilizer for thirty minutes on each of three successive days. The raw untreated milk was stored without treatment of any kind. The formolized milk was prepared by adding sufficient formaldehyde to raw clean milk to make 0.1 per cent. This is the quantity of formaldehyde that was used by Tice and Sherman<sup>6</sup> in their study of proteolysis of milk at the temperature of the laboratory.

The reinfected milk was prepared by the method of St. John and Pennington.<sup>7</sup> The organisms were precipitated from a portion of the raw milk by

<sup>3</sup> Pennington: *loc. cit.*

<sup>4</sup> Hepburn: *Journal of the Franklin Institute*, clxxii, p. 187, 1911.

<sup>5</sup> Pennington: *loc. cit.*

<sup>6</sup> Tice and Sherman: *Journ. of the Amer. Chem. Soc.*, xxviii, p. 189, 1906.

<sup>7</sup> St. John and Pennington: *Journ. of Inf. Dis.*, iv, p. 647, 1907.

centrifugalization at high speed in sterile glass tubes, which have the general shape of tubes for the centrifugal collection of urinary sediment, but have a capacity of 250 cc. The centrifuge carried eight such tubes and was run at a velocity of 3,000 revolutions per minute. A picture of the apparatus has been published.<sup>8</sup> The supernatant milk was removed by means of sterile pipettes; sterile physiological salt solution was added and mixed with the bacterial sediment; then the tubes were returned to the centrifuge and whirled. The supernatant solution was removed from the bacterial sediment with sterile pipettes, and the entire procedure was repeated several times in order to obtain organisms as free as possible from milk serum. The organisms were then sown in a portion of the sterile milk at the temperature of the room; care was taken that the total count per cubic centimeter of the reinfected milk should be approximately the same as the total count per cc. of the raw untreated milk.

Each of the four samples—raw untreated, formolized raw, reinfected sterile and sterilized—was kept in a sterile flask in a mechanically refrigerated chill-room at 0°C. At intervals of one week, the contents of each flask were mixed intimately by thorough shaking, and a sample was withdrawn by means of a large sterile pipette, placed in a sterile flask and submitted to analysis—bacterial, zymochemical and chemical. The sterile sample was analyzed at the beginning and at the end of the experiment. Its sterility was demonstrated at both times by plating. Needless to remark, on the first analysis of the fresh samples, one analysis sufficed for both the raw untreated and the formolized raw milk, and one analysis for both the sterilized and the reinfected sterile milk.

The source, preparation and sampling of the cream were the same as in the case of the milk.

The milk was held at 0°C. for a maximum period of thirty-five days, the cream for a maximum period of twenty-eight days.

Bacterial and zymochemical studies were made on both milk and cream.

For the chemical analysis, the milk served for a study of changes in freezing point, lactose, acidity and distribution of the nitrogen, while the cream served for a study of the freezing point and of the lipins, including the determination of the various fat constants and of the quantity of lecithin.

#### *Chemical technique.*

The total nitrogen, casein nitrogen, and albumin and syntonin nitrogen, were determined by the method of Van Slyke and Hart<sup>9</sup> as modified in this laboratory.<sup>10</sup> The caseose nitrogen was determined in the filtrate from the

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<sup>8</sup> Hepburn: *Journal of the Franklin Institute*, clxxi, p. 595, 1911.

<sup>9</sup> Van Slyke and Hart: New York Agricultural Experiment Station, Geneva. Bulletin 215, 1902, p. 101.

<sup>10</sup> Pennington: *This Journal*, iv, p. 360, 1908; Hepburn: *Journal of the Franklin Institute*, clxxii, p. 390, 1911.

albumin and syntonin by the method of Bömer<sup>11</sup> for proteose nitrogen. The amino-acid nitrogen was determined on a separate portion of the milk by the method of Bigelow and Cook.<sup>12</sup> In all cases the actual determination of the nitrogen in its various forms was by the Gunning method.

The determination of total nitrogen including nitrate nitrogen, was carried out according to the modified Gunning method<sup>13</sup> of the Association of Official Agricultural Chemists, using salicylic acid and sodium thiosulphate.

The free ammoniacal nitrogen was determined according to Berg and Sherman.<sup>14</sup>

In the tabulated results (Table I) the data included as undetermined nitrogen may be taken as a measure of peptone nitrogen.

The percentage of each form of nitrogen in terms of the milk is given in the tables in Roman type, while each form is also expressed as per cent of the total nitrogen of the milk by means of italics. The latter mode of expression of nitrogen results is of great help in the study of proteolysis.

Acidity was determined by titrating 10 cc. of milk at room temperature with  $\frac{N}{10}$  sodium hydroxide, using phenolphthalein as the indicator. The results are expressed as cc. of  $\frac{N}{10}$  sodium hydroxide required by 100 cc. of milk.

The lactose was determined by the optical method, after clarification by means of acid mercuric nitrate.<sup>15</sup>

The lecithin was extracted as directed by Nerking and Haensel<sup>16</sup> and was burned by means of sodium peroxide, as described by Le Clerc and Dubois<sup>17</sup> and by Dubois.<sup>18</sup> The lecithin phosphoric anhydride was then determined volumetrically by solution of the ammonium phosphomolybdate in a known volume of standard alkali, and titration of the excess of alkali. The per cent of lecithin was calculated by multiplying the per cent of lecithin phosphoric anhydride by the factor 11.41.

The fat for the determination of fat constants was extracted from the cream by the method used in this laboratory for the extraction of fat from egg yolk.<sup>19</sup> The cream was mixed with several times its volume of 95 per cent alcohol, and the precipitate was collected on a filter and dried over cal-

<sup>11</sup> Bömer: *Zeitschr. f. anal. Chem.*, xxxiv, p. 562, 1895.

<sup>12</sup> Bigelow and Cook: *Journ. of the Amer. Chem. Soc.*, xxviii, p. 1485, 1906.

<sup>13</sup> U. S. Department of Agriculture, Bureau of Chemistry, Bulletin 107, revised, p. 8.

<sup>14</sup> Berg and Sherman: *Journ. of the Amer. Chem. Soc.*, xxvii, p. 124, 1905.

<sup>15</sup> U. S. Department of Agriculture, Bureau of Chemistry, Bulletin 107, revised, p. 118.

<sup>16</sup> Nerking and Haensel: *Biochem. Zeitschr.*, xiii, p. 348, 1908.

<sup>17</sup> Le Clerc and Dubois: *Journ. of the Amer. Chem. Soc.*, xxvi, p. 1108, 1904.

<sup>18</sup> Dubois: *ibid.*, xxvii, p. 729, 1905.

<sup>19</sup> Pennington: this *Journal*, vii, p. 115, 1910.

cium chloride in a desiccator. The filtrate was evaporated to dryness *in vacuo*, using a water bath as a source of heat; and the residue was combined with the precipitate, then extracted for two days in a Soxhlet extractor with freshly distilled petroleum ether of boiling point 40–60°C. The solvent was removed by distillation on the water bath, and the residue of butter fat was used for the study of the fat constants, which were determined by the methods of the Association of Official Agricultural Chemists.<sup>20</sup> The procedure of Hanus was used for the determination of the iodine number, while that of Leffmann and Beam served for the determination of the Reichert-Meissl number. The index of refraction was taken on an Abbe refractometer which was provided with a water jacket. The ester value was determined by difference by subtracting the acid value from the saponification number.

For the determination of the freezing point, the apparatus of Beckmann was used. The fixed point of the Beckmann thermometer was adjusted and determined by means of dimethylaniline.

#### *Zymochemical methods.*

For the detection of catalase 5 cc. of milk or cream and 0.3 cc. of  $\frac{M}{10}$  hydrogen peroxide were mixed in a sterile Erlenmeyer flask of 50 cc. capacity, provided with a sterile delivery tube which dipped beneath a eudiometer in a pneumatic trough. The oxygen evolved was thus collected over water by upward, wet displacement. The period of incubation was forty-eight hours at 37.5°C. The volume of oxygen was measured at room temperature, which was noted as well as the barometric pressure. The difference of level of the water within and without the eudiometer was disregarded. The volume of oxygen has been reduced to a temperature of 0°C. and to a pressure of 760 mm. of mercury for insertion in the tabulated results.

In the study of reductases, the reagents—methylene blue and methylene-blue-formaldehyde—were prepared and used as directed by Schardinger.<sup>21</sup> No attempt was made to obtain anaerobic conditions; the period of incubation was from twelve to twenty-four hours at 37.5°C. A bleaching of the lower portion of the solution during that period of time was considered proof of the presence of reductase, even though a blue ring remained on the surface of the substratum.

The oxidase reagents were tincture of guaiac U.S.P.<sup>22</sup> and trikresol—a 3 per cent aqueous solution. Ten cubic centimeters of milk and 1 cc. of the reagent were mixed and incubated at 37.5°C. for twelve to twenty-four

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<sup>20</sup> U. S. Dept. of Agric., Bureau of Chemistry, Bulletin 107, revised, pp. 131–142.

<sup>21</sup> Schardinger: *Zeitschr. f. Untersuchung der Nahrungs und Genussmittel*, v, p. 1113, 1902.

<sup>22</sup> *Pharmacopoeia of the United States of America*, 8th Decennial Revision, p. 467.

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hours. The production of a blue color by the guaiac and of a violet or purple color by the trikresol during the period of incubation was considered to indicate the presence of an oxidase.

In the tabulated results, the presence of either of the reductases or of either of the oxidases is designated by a plus sign (+) in the proper column, while the absence of the enzyme is recorded by a minus sign (−) in the proper column.

### *Bacteriological technique.*

To determine the total number of organisms per cc., plain nutrient agar was sown with the milk or cream.

To determine the number of acid-formers present, plates of litmus lactose agar were sown and a count was made of all acid-forming colonies.

To determine the number of anaerobes and facultatives present, plates of plain nutrient agar were sown according to Wright's anaerobic plate method.

In every case the dilutions were carried high enough to insure between one hundred and two hundred organisms on each plate, and duplicate plates were always made of every dilution used.

The plates were incubated at 37°C. for two days, 20°C. for five days, and 0°C. for four weeks. A Stewart counting chamber and a 1½ inch lens were used for counting; the results were recorded as directed by the American Public Health Association.<sup>22</sup>

To estimate the number of liquefying organisms present, sowings were made in nutrient gelatin; the plates were incubated at 20°C. for five days and the number of organisms liquefying the medium were counted and the results recorded as stated above.

### *Isolation and identification of organisms.*

For the isolation, identification and comparative rate of growth of the organisms in the milk and the cream, plates were selected where the colonies had sufficient space for free development. All the colonies were counted, and those with like cultural characteristics were grouped and studied as to their morphology and relative rate of growth and appearance of the agar streak, when incubated at 37°C. (incubator), 22°C. (room), and 20°C. (refrigerator), respectively. The optimum temperature for each organism isolated was used for the further study of its morphology and biochemical characteristics. Characteristic growth on plates of plain nutrient agar, litmus lactose agar, and nutrient gelatin, respectively, were studied, also growth in stab culture on nutrient agar and nutrient gelatin. The characteristic growth and reaction produced by the organisms in neutral bouillon and in litmus milk were noted, as were indol production, reduction

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<sup>22</sup> American Public Health Association: *Standard Methods for the Examination of Water and Sewage*, 2nd edition, 1912, p. 79.



of nitrate to nitrite, digestion of casein and of gelatin, chromogenicity and fluorescence. The aerobic, anaerobic and facultative properties were studied as well as morphology, motility, arrangement of flagella, spore-formation and reaction toward the Gram stain.

#### THE CHEMICAL CHANGES IN THE MILK DURING HOLDING.

##### *The distribution of the nitrogen.*

On the first analysis of the reinfected sterile and the sterilized milks, it was seen that the heat during sterilization had given rise to a partial coagulation of the albumin; this coagulated albumin precipitated with the casein in the analytic separation of the two proteins, hence the casein nitrogen was high and the albumin and syntonin nitrogen low for a fresh milk. The milk had also been slightly concentrated by sterilization as shown by the total nitrogen which was slightly higher in the sterile reinfected and the sterilized milks than in the raw untreated and the formolized raw milks.

In the *raw, untreated* milk, the casein nitrogen underwent a progressive decrease; the albumin and syntonin nitrogen was almost the same at the beginning and end of the experiment, its fluctuations during the intermediate analyses being doubtless due to formation and decomposition of metaprotein at the expense of the casein. The digestion of the casein must account for the large increase in caseose nitrogen during the latter half of the period of holding. That the caseoses may undergo digestion is shown by the progressive decrease in that form of nitrogen during the first half of the experiment. The amino-acid nitrogen showed a marked tendency to increase at the expense of the protein nitrogen. During the proteolysis, peptones were doubtless formed as is shown by the variations in the data listed as "undetermined nitrogen," which is a measure of peptone nitrogen by difference. The quantity of free ammoniacal nitrogen was but slight and fluctuated wildly. Nitrates were not formed during holding, for, at the end of the experiment, the total nitrogen and total nitrogen including nitrates were practically the same, hence, nitrogen fixers were absent from the milk.

Ravenel, Hastings and Hammer<sup>24</sup> analyzed a clean milk and a fair grade of commercial milk which had been held at low tempera-

<sup>24</sup> Ravenel, Hastings and Hammer: *Journ. of Inf. Dis.*, vii, p. 38, 1910.

tures for a period of 203 days. In addition to the total nitrogen, they record the "water-soluble" nitrogen which was obtained by diluting the milk with water, adding "a small amount" of acetic acid at the temperature of the water bath, filtering and determining the nitrogen content of the filtrate. During holding, the soluble nitrogen—expressed as per cent of the total nitrogen—became higher than in fresh milk, being 17.97 in the clean milk and 22.38 in the commercial milk kept at  $-9^{\circ}\text{C}.$ ; and over 72 in both milks kept at  $0^{\circ}\text{C}.$  In the latter experiments the total nitrogen decreased to a marked degree; the loss is ascribed to the liberation of elementary nitrogen. These investigators refer the proteolysis at  $0^{\circ}\text{C}.$  to bacterial action; that at  $-9^{\circ}\text{C}.$  to the action of the native milk enzyme galactase. Since their method of chemical analysis differed widely from that used in the present research the results obtained in the two studies are not strictly comparable.

In the *reinfected, sterile* milk, the casein nitrogen decreased progressively but to a far less degree than did the casein nitrogen of the raw untreated milk. The albumin and syntonin nitrogen varied within narrow limits and showed no marked change during the period of keeping. The caseose nitrogen showed a progressive increase most marked during the last third of the experiment. The amino-acid nitrogen increased to some extent, and the peptone nitrogen tended to decrease. Since, on the final analysis, the total nitrogen and the total nitrogen including nitrates were the same, nitrates were not formed during holding. Hence, nitrogen fixers were not present in the milk. The amount of free ammoniacal nitrogen was small and apparently tended to decrease.

In the *formolized, raw* milk, the casein nitrogen remained practically constant during the entire period of holding. The albumin and syntonin nitrogen tended to decrease and the caseose nitrogen to increase. The peptone nitrogen showed a tendency to decrease, and the amino-acid nitrogen a tendency to increase. The free ammoniacal nitrogen was a negligible quantity. In this connection it should be mentioned that Tice and Sherman,<sup>25</sup> during a study of formolized raw milk held at room temperature for periods as long as thirty-seven months, noted that "the albumin was largely digested before the original amount of casein was appreciably reduced." Hence, at the temperature of the chill-room and of the

<sup>25</sup> *Loc. cit.*

room, the same type of proteolysis occurs in formolized raw milk, and is produced mainly, if not entirely, by galactase—a native milk enzyme; possibly proteolytic enzymes derived from the dead bacteria, may also participate in the digestion of the protein. On the other hand, Sherman, Berg, Cohen and Whitman<sup>26</sup> reported that the free ammoniacal nitrogen increased in formolized raw milk kept in the room at 15°C. for three months, while in the present research the free ammoniacal nitrogen was found to be an absolutely negligible quantity in formolized raw milk held at 0°C.

In the *sterilized* milk, during the entire period of storage, the changes in the distribution of the nitrogen were but slight, and, on the whole, lie within the limits of analytic error.

The study of the nitrogen results leads to the following conclusions. The proteolysis of the casein is, primarily, of bacterial origin, since it occurred in the reinfected sterile milk, but not in the formolized raw milk. The digestion of the albumin and syn-tonin is, primarily, due to native enzymes of the milk, since it took place in the formolized raw but not in the reinfected sterile milk. In raw untreated milk, however, the native enzymes and bacterial flora act in combination in giving rise to more rapid proteolytic changes, since in the same period of time—five weeks—over twice as much casein was digested in the raw untreated milk as in the reinfected sterile milk. The general trend of the proteolysis, enzymic, bacterial and combined, is toward a tryptic digestion, that is, the passage through caseose and peptone to amino-acids which accumulate as the period of holding lengthens. The changes in the ammoniacal nitrogen are negligible.

#### *Acidity.*

The acidity of the *raw, untreated* milk increased more or less progressively to the highest values of the entire series of experiments. In the *reinfected, sterile* milk, the acidity increased progressively and finally attained values which were second only to those obtained in the raw untreated milk. In the *formolized, raw* milk the acidity first increased, then decreased to a value which remained fairly constant to the very end of the experiment. The initial rise was possibly due to the bacterial enzymes of the dead

<sup>26</sup> Sherman, Berg, Cohen and Whitman: This *Journal*, iii, p. 171, 1907.

TABLE I.

*Chemical changes in raw and treated clean milk kept at 0° C.*

(The per cent of the various forms of nitrogen in the milk are printed in Roman; the various forms of nitrogen are also printed as per cents of the total nitrogen in italics. Lactose is expressed as per cent and acidity as cc. of  $\frac{N}{10}$  NaOH required to neutralize 100 cc. of milk.)

PERIOD OF HOLD- ING AT 0°C.	DISTRIBUTION OF THE NITROGEN							LACTOSE	ACIDITY	FREEZING POINT
	Total N*	Casein N	Albumin and syntonin N	Caseose N	Amino-acid N	Undetermin- ed N	Free ammon- iacal N			
<i>Raw untreated milk.</i>										
<i>days</i>										<i>deg. C.</i>
Fresh	0.585	0.457	0.067	0.033	0.007	0.019	0.00047	5.3	18.0	-0.550
		78.12	11.79	5.64	1.20	3.25	0.080			
7	0.571	0.432	0.084	0.018	0.018	0.019	0.00046	5.2	16.5	-0.540
		75.66	14.71	3.15	3.15	3.33	0.080			
14	0.566	0.377	0.081	0.000	0.032	0.076	0.00000	3.8	32.7	-0.565
		66.61	14.31	0.00	5.65	13.43	0.000			
21	0.569	0.335	0.069	0.167	0.033	†0.035	0.00075	3.7	30.8	-0.595
		58.88	12.13	29.35	5.80	6.15	0.131			
28	0.537	0.295	0.053	0.161	0.022	0.006	0.00113	3.3	46.5	-0.585
		54.93	9.87	29.98	4.10	1.12	0.210			
35	0.572	0.286	0.066	0.136	0.044	0.040	0.00040	3.5	56.0	-0.630
		50.00	11.54	23.78	7.69	6.99	0.069			
<i>Reinfected sterile milk.</i>										
Fresh	0.608	0.528	0.018	0.029	0.010	0.023	0.0029	4.5	25.8	-0.735
		86.84	2.96	4.77	1.64	3.78	0.477			
7	0.595	0.516	0.021	0.016	0.030	0.012	0.00356	5.1	26.0	-0.550
		86.72	3.53	2.69	5.04	2.02	0.598			
14	0.585	0.522	0.015	0.029	0.033	†0.014	0.00410	4.2	32.0	-0.560
		89.23	2.56	4.96	5.64	2.39	0.701			
21	0.590	0.507	0.013		0.027		0.00210	4.4	30.8	-0.585
		85.93	2.20		4.58		0.356			
28	0.592	0.463	0.015	0.080	0.014	0.020	0.00168	3.3	41.3	-0.570
		78.21	2.53	13.51	2.36	3.38	0.284			
35	0.596	0.444	0.023	0.105	0.026	†0.002	0.00108	3.5	51.8	-0.680
		74.50	3.86	17.62	4.36	0.34	0.181			

\*Total nitrogen, including nitrate nitrogen, at 35th day of holding: raw untreated milk, 0.565; reinfected sterile milk, 0.595.

†In this set of analyses, the sum of the nitrogenous constituents determined exceeds the total nitrogen of the milk by this amount.

TABLE I—Continued.

PERIOD OF HOLD- ING AT 0°C.	DISTRIBUTION OF THE NITROGEN							LACTOSE	ACIDITY	FREEZING POINT
	Total N	Casein N	Albumin and syntonin N	Caseose N	Amino-acid N	Undeterm- ined N	Free ammon- iacal N			
Formolized raw milk.										
days										deg. C.
Fresh	0.585	0.457	0.069	0.033	0.007	0.019	0.00047	4.8	18.0	-0.550
	78.12	11.79	5.64	1.20	3.25	0.080				
7	0.573	0.450	0.056	0.023	0.015	0.029	0.00060	5.1	19.0	-0.660
	78.53	9.77	4.01	2.62	5.06	0.010				
14	0.564	0.463	0.044	0.031	0.026	0.000	0.00000	4.4	27.8	-0.650
	82.09	7.80	5.50	4.61	0.00	0.000				
21	0.550	0.465	0.051		0.025		0.00000	5.0	19.8	-0.655
	84.55	9.27		4.55		0.000				
28	0.561	0.465	0.032	0.048	0.012	0.004	0.00000	4.8	21.0	-0.655
	82.89	5.70	8.56	2.14	0.71	0.000				
35	0.602	0.498	0.033	0.047	0.019	0.005	0.00000	5.3	21.5	-0.680
	82.72	5.48	7.81	3.16	0.83	0.000				
Sterilized milk.†										
Fresh	0.608	0.528	0.018	0.029	0.010	0.023	0.0029	4.5	25.8	-0.735
	86.84	2.96	4.77	1.64	3.78	0.477				
35	0.637	0.547	0.012	0.027	0.027	0.024	0.00298	4.6	26.0	-0.615
	85.87	1.88	4.24	4.24	3.77	0.468				

†No analyses made during first four weeks of holding.

organisms, which had been killed by the formaldehyde. The subsequent decrease in acidity may be ascribed to either a neutralization or further decomposition of the lactic acid. In the *sterilized* milk the acidity remained constant during the entire period of holding.

Ravenel, Hastings and Hammer<sup>27</sup> report a decrease in the acidity of a clean milk and of a fair grade of commercial milk, held at -9°C. for a period of 203 days; and an increase, decidedly progressive, in the acidity of both the clean milk and the commercial milk held at 0°C. for that period of time.

<sup>27</sup> *Loc. cit.*

*Lactose.*

The lactose content of the *raw, untreated* milk decreased progressively, the greatest loss occurring during the earlier portion of the period of storage. In the *reinfected, sterile* milk the decrease in lactose tended to parallel that of the raw untreated milk. The tendency was for the lactose to decrease but little, if at all, in the *formolized, raw* milk; a similar tendency was noted by Tice and Sherman<sup>28</sup> in raw formolized milk kept at the temperature of the laboratory. The *sterilized* milk showed no change in lactose content during the period of holding. The fermentation of the lactose with the formation of lactic acid was then largely, if not exclusively, due to the activity of bacteria.

*Freezing point.*

The decomposition of the constituents of the milk, especially the carbohydrate and the protein, with the formation of several small molecules from one large molecule must give rise to a higher molecular concentration of the solutes of the milk and, therefore, should be accompanied by a depression, or lowering, of the freezing point of the milk. The analytic findings are in perfect harmony with this theory. In the *raw, untreated* milk, the proteolysis and the fermentation of the lactose were accompanied by a lowering of the freezing point, which tended to be a progressive change. The changes in distribution of the nitrogen in the *formolized, raw* milk and the changes in protein and lactose in the *reinfected, sterile* milk, were likewise accompanied by lowerings of the freezing point.

## THE CHEMICAL CHANGES IN THE CREAM DURING HOLDING.

*The fat constants.*

The iodine number remained practically unchanged in all the experiments. The index of the refraction underwent no change in any of the samples. The Reichert-Meissl number showed no marked change.

In the *raw, untreated* cream, the saponification number, Hehner number and acid value seemingly increased progressively; the greatest rise in the saponification number occurred during the

<sup>28</sup> *Loc. cit.*

first week. In the *reinfected, sterile* cream, the saponification number showed an increase, followed by progressive decreases. The Hehner number and the acid value increased, with a tendency to do so progressively, the greatest increase being during the first week. In the *formolized, raw* cream the saponification number increased, especially during the first week, the Hehner number also increased with a tendency to do so progressively, although the greatest increment was during the first week. The acid value increased to a very slight extent. In the *sterilized* cream, the saponification number and the Hehner number increased, while the acid value suffered a slight decrease.

If the values of the saponification and Hehner numbers, and of the acid value obtained on the initial analysis, be compared with the highest values obtained in the subsequent analyses of each series, it will be observed that the greatest increase in acid value occurred in the reinfected sterile cream, the least in the formolized raw cream, with the raw untreated cream occupying an intermediate position. This would tend to show that the fat-splitting is of bacterial origin rather than due to enzymes of the cream.

The simultaneous increase in both Hehner and saponification numbers occurred even in the sterilized cream, showing that this reaction may depend simply on oxidation and the fine state of division of the butter-fat, possibly aided by a thermostable inorganic catalyst. Since this change was most pronounced in the formolized raw cream, native enzymes of the cream must also play a prominent rôle in its production.

The progressive decreases in the saponification number, accompanied by an increase in the Hehner number, in the reinfected sterile cream denote a type of fat decomposition, different from that observed in the sterilized, formolized raw, and raw untreated cream. Apparently, the raw untreated cream represents the resultant of the bacterial changes, revealed by the reinfected sterile cream, of oxidation and catalytic changes seen in the sterilized cream, and of the enzymic changes occurring in the formolized raw cream. The fine state of division of the butter-fat in the cream also, doubtless, plays a rôle in all the samples by exposing a large surface for oxidation.

TABLE II.

*Chemical changes in raw and treated clean cream kept at 0°C.*

PERIOD OF HOLD- ING AT 0°C.	Iodine num- ber	Saponification number	Acid value	FAT CONSTANTS				Index of re- fraction at 35°C.	LECITHIN	FREEZING POINT
				Ester value	Per cent of free acid as oleic	Hegner num- ber	Reichert Massei num- ber			
<i>Raw untreated cream.</i>										
days								per cent		deg. C.
Fresh	32.0	206.0	0.5	205.5	0.25	76.8	31.1	1.4545	0.1159	-0.543
7	32.5	228.7	1.8	226.9	0.91	81.75	34.6	1.4546	0.1061	-0.620
14	33.1	221.2	1.2	220.0	0.60	85.0	31.0	1.4543	0.0917	-0.685
21	33.3	229.8	1.3	228.5	0.65	85.6	33.9	1.4543	0.1220	-0.720
28	31.4	226.5	2.1	224.4	1.06	83.0	33.0	1.4548	0.1388	-0.712
<i>Reinfected sterile cream.</i>										
Fresh	32.1	213.2	0.7	212.5	0.35	79.82	26.4	1.4546	0.1067	-0.565
7	33.1	228.6	2.6	226.0	1.31	83.6	34.7	1.4544	0.0967	-0.570
14	32.9	220.7	2.5	218.2	1.26	81.7	34.0	1.4544	0.1104	-0.600
21	33.4	183.9	2.8	181.1	1.41	85.4	30.0	1.4543	0.1106	-0.615
<i>Formolized raw cream.</i>										
Fresh	32.0	206.0	0.5	205.5	0.25	76.8	31.1	1.4545	0.1159	-0.543
7	32.8	233.7	0.52	233.2	0.26	83.6	27.4	1.4542	0.1249	-0.665
14	33.0	232.9	0.43	232.5	0.22	85.4	22.0	1.4542	0.1342	-0.655
21	32.5	238.6	0.33	238.3	0.17	85.9	35.5	1.4544	0.1529	-0.650
28	30.9	229.3	0.53	228.8	0.27	84.0	35.4	1.4535	0.1222	-0.660
<i>Sterilized cream.*</i>										
Fresh	32.1	213.2	0.7	212.5	0.35	79.82	26.4	1.4546	0.1067	-0.565
28	33.0	235.2	0.3	234.9	0.15	85.6		1.4551	0.1158	-0.622

\*No analyses made during first three weeks of holding.



*The lecithin.*

This lipin apparently has not been decomposed in any of the four series of experiments.

*Freezing point.*

The *raw, untreated* cream showed a progressive lowering of the freezing point, as did the *reinfected, sterile* cream, though to a less degree. The *formolized, raw* cream showed a depression during the first week, then remained fairly constant. The *sterilized* cream showed a slight depression, but less than took place in the other samples. These changes were probably due to digestion of the protein and lactose of the cream, rather than to fat decomposition, for the Reichert-Meissl number, which may be accepted as a fair measure of soluble fatty acids, had undergone no marked increase; therefore, no soluble decomposition products of the butter-fat had been formed to exert an influence on the freezing point.

## ENZYMES OF THE MILK AND CREAM.

The results of the zymochemical experiments have been collected in Table III. In the *raw, untreated* milk and cream, reductases which attack methylene blue were apparently absent from both the fresh milk and the fresh cream, but were invariably present after the first week of holding at 0°C. Reductases which act upon methylene blue, plus formaldehyde, were present in both the fresh milk and the fresh cream and retained their activity throughout the period of storage. Oxidases which give rise to the oxidation of trikresol were always present in both the milk and the cream, while oxidases which are reactive toward guaiac were found in but three of the six milk analyses and in but two of the five cream analyses. In the *reinfected, sterile* milk and cream, reductases which destroy the color of methylene blue, as well as reductases which decolorize methylene blue, plus formaldehyde, were present in all the samples of milk save that tested after holding for one week, and were invariably present in the cream. Oxidases which produce oxidation of trikresol were always present in both the milk and the cream, while oxidases which cause a coloration of guaiac were always absent from the cream and were found only in the fresh

milk. In the *formolized*, raw milk and cream, reductases which decolorize methylene blue were absent from the fresh milk and from the final sample (held for thirty-five days at 0°C.) but were present in the other milk samples and invariably present in the cream. Reductases, which act on methylene blue plus formaldehyde, were always present in the cream and occurred in the fresh milk but were not found in any of the samples of milk which had been held at 0°C. for one or more weeks. Oxidases which attack trikresol were invariably present in both the milk and the cream, while oxidases which are reactive toward guaiac were invariably absent from both the milk and the cream.

Unfortunately, the data on catalase are incomplete. However, this enzyme apparently occurs as a true milk enzyme and is also secreted by the microorganisms. The raw untreated milk was always able to liberate more oxygen from hydrogen peroxide than was either the reinfected sterile or the formolized raw milk, yet this rule did not hold good in the case of the cream. At the end of two weeks' storage at 0°C. the cream showed greater catalytic activity than did the milk, but the reverse was true at the end of the third week.

To sum up, reductases which attack methylene blue were normal constituents of the raw untreated, reinfected sterile, and formolized raw samples of milk and of cream. Reductases which decolorize methylene blue in the presence of formaldehyde were invariably present in all three kinds of cream, were normally present in the raw untreated milk and in the reinfected sterile milk, and were usually absent from the formolized raw milk. It is, therefore, probable that the aldehyde reductases of the milk were of bacterial origin.

Oxidases, which give rise to an oxidation of trikresol, were invariably present in both milk and cream—raw untreated, reinfected sterile, and formolized raw. Oxidases, which produce a color with guaiac, were present in about half the experiments on raw untreated milk and cream, and were absent from all the other samples with the single exception of the fresh reinfected sterile milk.

These results point to the conclusion that the simple reductases and the trikresol oxidases of the milk may be enzymes native to the milk and may also be of bacterial origin. The aldehyde reductase

TABLE III.  
*Enzymes of raw and treated milk and cream kept at 0°C.*  
(The presence or absence of enzymes is expressed by the signs + and - respectively, except in the case of catalase where the figures represent cc. of oxygen evolved.)

SAMPLE NUMBER AND DESCRIPTION	PERIOD IN DAYS OF HOLDING SAMPLE AT 0°C	RAW UNTREATED					FORMOLIZED RAW				REINFECTED STERILE			
		CATALASE	REDUCTASE Methylene blue	REDUCTASE Methylene blue plus formal- hyde	OXIDASES Guaiaac	OXIDASES Trikresol	CATALASE	REDUCTASE Methylene blue	REDUCTASE Methylene blue plus formal- hyde	OXIDASES Guaiaac	OXIDASES Trikresol	CATALASE	REDUCTASE Methylene blue	REDUCTASE Methylene blue plus formal- hyde
167 Milk.....	fresh		-	+	+	+		+	+	+	+		+	+
	7	2.65	+	+	+	+	1.60	+	+	+	+	0.55	+	+
	14	9.85	+	+	+	+	7.70	+	+	+	+	9.00	+	+
	21	13.40	+	+	+	+		+	+	+	+		+	+
	28		+	+	+	+		+	+	+	+		+	+
	35		+	+	+	+		+	+	+	+		+	+
168 Cream.....	fresh		-	+	+	+		+	+	+	+		+	+
	7	2.70	+	+	+	+	2.80	+	+	+	+	6.65	+	+
	14	3.80	+	+	+	+	5.70	+	+	+	+		+	+
	21		+	+	+	+		+	+	+	+		+	+
	28		+	+	+	+		+	+	+	+		+	+

of the milk was probably of bacterial origin, and apparently the guaiac oxidases arose from the same source. In the cream both varieties of reductase and the trikresol oxidases were apparently native enzymes and were also secreted by the microorganisms, while the guaiac oxidases were probably of bacterial origin. The catalase of both the milk and the cream was a true milk enzyme and was also due to the activity of microorganisms. All five enzymes—the two varieties of oxidase, the two varieties of reductase, and the catalase—retained their power to act as catalytic agents in spite of the prolonged exposure to a temperature of 0°C.

#### THE BACTERIAL CHANGES IN THE MILK DURING HOLDING.

Conn and Esten<sup>29</sup> report three experiments in which milk was kept at 1°C. for periods as long as 42 days, bacteriological analyses being made at intervals of 2 days to 9 days. During the first 6 or 8 days of holding, scarcely any bacterial development occurred, then the organisms increased steadily until very great numbers were present. Since the usual lactic acid organisms were not in the majority, the milk did not curd. A comparatively large number of liquefying organisms, and of neutral organisms, which produce neither acid nor alkali, were found in the milk. There was also a tendency for certain organisms to disappear during the period of holding.

Ravenel, Hastings and Hammer,<sup>30</sup> studied bacterial growth in milk held at low temperatures. The milk was of two kinds, "barn milk," the best milk obtainable, and "dairy milk," a fair commercial article. One sample of each kind of milk was kept at -9°C., and at 0°C., for a maximum period of 203 days, analyses being made at varying intervals. Up to and including 160 days, plates of lactose agar were sown and incubated at 37°C.; plates of plain gelatin were sown and incubated at 12 to 15°C. up to and including 203 days. During holding at -9°C., the number of organisms growing on agar at 37°C. remained fairly constant in both kinds of milk, the variations being insufficient to permit of any definite conclusions. The number of organisms developing

<sup>29</sup> Conn and Esten: *Sixteenth Annual Report of the Storrs Agricultural Experiment Station*, 1904, p. 27.

<sup>30</sup> *Loc. cit.*

on gelatin at 12 to 15°C. was fairly constant in the barn milk, but decreased considerably and with a marked progressive tendency in the dairy milk.

During holding at 0°C., the organisms growing on agar at 37°C. underwent a quiescent stage in both kinds of milk for six days, then increased progressively, in the barn milk to the end of the period of holding, in the dairy milk up to and including seventy-four days, afterward decreasing progressively. The organisms developing on gelatin at 12° to 15°C. were characterized by progressive increases to a maximum count followed by decreases; the maximum was attained in the dairy milk much earlier than in the barn milk.

In the *raw, untreated* milk on the plates incubated at 37°, the total count per cc. increased progressively up to the twenty-first day, then dropped rapidly and progressively. The anaerobes and facultatives rose during the first week, then fell; the maximum count, however, was at 21 days, after which a rapid progressive decrease occurred. The acid formers underwent a progressive increase up to the twenty-first day, then a rapid progressive decrease took place. On the plates incubated at 20°, the total count per cc. showed a progressive increase throughout the period of holding. There was a decided tendency for the anaerobes and facultatives to increase, the highest counts being at 21 and 28 days. The acid formers and the liquefiers increased progressively. On the plates incubated at 0°, the total count per cc. showed progressive increases and reached a maximum at 35 days. The anaerobes and facultatives tended to increase, and while this increase was not absolutely progressive, the highest counts were at 21 and 35 days. The acid formers were characterized by an increase, which, on the whole, was progressive.

In the *reinfected, sterile* milk, on the plates incubated at 37°, the total count per cc. rose to a maximum at 7 days, then suffered a progressive decrease throughout the experiment. The changes in the anaerobes and facultatives paralleled those of the total count, the maximum being at 7 days, after which there was a progressive decrease until no growth was obtained on the final analysis. The changes in the acid formers ran parallel to those of the total count and of the anaerobes and facultatives, the maximum at 7 days, then a progressive decrease to the end of the period of holding. On the plates incubated at 20°, the total count per cc. tended to rise

progressively, the maximum being at 28 days. The number of anaerobes and facultatives showed wide fluctuations, the greatest number being found at 14 and 35 days. The acid formers showed a decided trend to increase progressively, the maximum being at 28 days. The liquefiers increased progressively throughout the experiment. On the plates incubated at 0°, the total count per cc. rose progressively throughout the experiment. The anaerobes and facultatives and the acid formers showed a general tendency to increase progressively, reaching their highest counts at 28 days.

While a few organisms, including anaerobes and facultatives and acid formers, were present in the fresh *formolized raw* milk, yet in the subsequent analyses the plates were almost invariably sterile. The formolized raw milk of Tice and Sherman,<sup>31</sup> which was kept at the temperature of the laboratory, at times contained liquefying cocci, which formed yellow colonies, as well as cocci and bacilli, which formed white colonies.

The *sterile* milk retained its sterility throughout the period of holding, as was demonstrated by platings made on the initial and final analyses.

#### *Comparison of the raw and reinfected milk.*

As a general rule, the total counts on the raw untreated milk were higher than on the reinfected sterile milk at all temperatures of incubation through the experiment, although the initial total count was practically the same. The initial counts of anaerobes and facultatives were fairly close in the two kinds of milk; during the first 2 weeks the reinfected sterile milk showed the highest count at all temperatures of incubation; at 3 weeks the raw untreated milk had the higher count at all temperatures of incubation; the raw untreated milk also continued to have the higher count at 37° throughout the rest of the experiment.

The initial count of acid formers differed in the two experiments, so the counts throughout the experiment can scarcely be compared. It may be noted that the relation of the acid formers to the total count varied throughout both series. At times the number of liquefiers was higher in the raw untreated milk, at times in the reinfected sterile milk.

<sup>31</sup> *Loc. cit.*

*Influence of temperature on the organisms.*

On comparison of the counts, incubated at the three temperatures on the same analysis, the total count per cc. in the raw untreated milk had either 20° or 0° as the optimum temperature after the first week, in the reinfected sterile milk, 20° after the first week and 0° at the fifth week.

The anaerobes and facultatives in the raw untreated milk passed from an optimum temperature of 37° through 20° to 0°, while in the reinfected sterile milk the optimum growth was at either 20° or 0°.

The acid formers almost invariably had a maximum count on the plates grown at 20°.

*Ratio of increase of the organisms.*

The maximum count obtained during the period of holding with each group of organisms at each temperature of incubation is always compared with the count obtained with that group of organisms at the same temperature at the beginning of the experiment, using the latter figure as unity in the ratio.

RAW, UNTREATED MILK. *Total count per cc.* At 37° the maximum was attained at 21 days and was 463.9 times the count on the fresh milk. At 20° the maximum was at 35 days and the ratio of increase 1:3563.2; at 0° the maximum was at 35 days and the ratio of increase 1:290909.1. *Anaerobes and facultatives.* At 37° the maximum was at 21 days and the ratio of increase 1:118.9, at 20° the maximum was at 21 days and the ratio 1:340.0, at 0° the maximum was at 35 days and the ratio 1:533.3. *Acid formers.* At 37° the maximum was at 21 days and the ratio of increase 1:1913.0, at 20° the maximum was at 35 days and the ratio 1:12258.1, and at 0° the maximum was at 35 days and the ratio 1:357894.7. The maximum count of liquefiers was at 21 days and the ratio of increase 1:19000.0.

REINFECTED, STERILE MILK. *Total count per cc.* At 37° the maximum was at 7 days and the ratio of increase was 1:13.0, at 20° the maximum was at 28 days and the ratio 1:1437.5, at 0° the maximum was at 35 days and the ratio 1:19200.0. *Anaerobes and facultatives.* At 37° the maximum was at 7 days and the ratio of increase 1:34.6, at 20° the maximum was at 28 days and the ratio 1:505.9, at 0° the maximum was at 28 days and the ratio 1:454.5. *Acid formers.* At 37° the maximum was at 7 days and the ratio of increase 1:9.9, at 20° the maximum was at 28 days and the ratio 1:3035.7, and at 0° the maximum was at 28 days and the ratio 1:13333.3. The maximum count of liquefiers was at 35 days and the ratio of increase 1:5000.0.

TABLE IV. *Bacterial co*  
(The figures in Roman represent b

PERIOD OF KEEPING IN DAYS	PLATES INCUBATED AT °C	RAW UNTREATED MILK			
		Total count per cc.	Anaerobes and Facultatives per cc.	Acid Formers per cc.	Liquefiers per cc.
Fresh.....	37	97,000 <i>1</i>	18,500 <i>1</i>	11,500 <i>1</i>	
	20	87,000 <i>1</i>	10,000 <i>1</i>	15,500 <i>1</i>	1,000 <i>1</i>
	0	1,100 <i>1</i>	4,500 <i>1</i>	475 <i>1</i>	
7.....	37	4,800,000 <i>49.5</i>	800,000 <i>43.2</i>	3,300,000 <i>28.7</i>	
	20	9,500,000 <i>109.2</i>	360,000 <i>36.0</i>	4,700,000 <i>303.2</i>	1,500,000 <i>1500.0</i>
	0	10,000,000 <i>9090.9</i>	240,000 <i>53.3</i>	4,400,000 <i>9263.2</i>	
14.....	37	13,000,000 <i>134.0</i>	85,000 <i>4.6</i>	7,600,000 <i>680.9</i>	
	20	87,000,000 <i>1000.0</i>	150,000 <i>15.0</i>	46,000,000 <i>2967.7</i>	2,700,000 <i>2700.0</i>
	0	66,000,000 <i>60,000.0</i>	90,000 <i>20.0</i>	25,000,000 <i>52,631.6</i>	
21.....	37	45,000,000 <i>463.9</i>	2,200,000 <i>118.9</i>	22,000,000 <i>1913.0</i>	
	20	180,000,000 <i>2069.0</i>	3,400,000 <i>340.0</i>	120,000,000 <i>7741.9</i>	19,000,000 <i>19,000.0</i>
	0	220,000,000 <i>200,000.0</i>	2,000,000 <i>444.4</i>	140,000,000 <i>294,736.8</i>	
28.....	37	170,000 <i>1.8</i>	20,000 <i>1.1</i>	80,000 <i>7.0</i>	
	20	180,000,000 <i>2069.0</i>	3,300,000 <i>330.0</i>	110,000,000 <i>7096.8</i>	
	0	150,000,000 <i>136,363.6</i>	1,200,000 <i>266.6</i>	67,000,000 <i>141,052.6</i>	
35.....	37	14,000 <i>0.14</i>	4,000 <i>0.22</i>	8,000 <i>0.70</i>	
	20	310,000,000 <i>3563.2</i>		190,000,000 <i>12,258.1</i>	15,000,000 <i>15,000.0</i>
	0	320,000,000 <i>290.909.1</i>	2,400,000 <i>533.3</i>	170,000,000 <i>357,894.7</i>	



*in milk kept at 0°C.*

*in Italic represent ratios of increase.)*

REINFECTED STERILE MILK				FORMOLIZED RAW MILK
Total count per cc.	Anaerobes and Facultatives per cc.	Acid Formers per cc.	Liquefiers per cc.	Total count per cc.
100,000	26,000	91,000		400
<i>1</i>	<i>1</i>	<i>1</i>		
160,000	17,000	56,000	5,000	140
<i>1</i>	<i>1</i>	<i>1</i>	<i>1</i>	
12,500	4,400	9,000		0
<i>1</i>	<i>1</i>	<i>1</i>		
1,300,000	900,000	900,000		0
<i>13.0</i>	<i>34.6</i>	<i>9.9</i>		
1,500,000	600,000	1,000,000	20,000	0
<i>9.4</i>	<i>35.3</i>	<i>17.9</i>	<i>4.0</i>	
58,000		27,000		0
<i>4.6</i>		<i>3.0</i>		
900,000	120,000	330,000		0
<i>9.0</i>	<i>4.6</i>	<i>3.6</i>		
10,000,000	1,400,000	9,000,000	4,500,000	0
<i>62.5</i>	<i>82.4</i>	<i>160.7</i>	<i>900.0</i>	
9,100,000	460,000	4,600,000		0
<i>728.0</i>	<i>104.5</i>	<i>511.1</i>		
200,000	50,000	120,000		5
<i>2.0</i>	<i>1.9</i>	<i>1.3</i>		
72,000,000	330,000	54,000,000	12,000,000	
<i>450.0</i>	<i>19.4</i>	<i>964.3</i>	<i>2400.0</i>	
67,000,000	550,000	32,000,000		
<i>5400.0</i>	<i>125.0</i>	<i>3711.1</i>		
29,000	10,000	27,000		0
<i>0.29</i>	<i>0.38</i>	<i>0.30</i>		
230,000,000	8,600,000	170,000,000	14,000,000	0
<i>1437.5</i>	<i>505.9</i>	<i>3035.7</i>	<i>2800.0</i>	
180,000,000	2,000,000	120,000,000		0
<i>14,400.0</i>	<i>454.5</i>	<i>13,333.3</i>		
3,000	0	1,000		
<i>0.03</i>		<i>0.01</i>		
160,000,000	1,300,000	72,000,000	25,000,000	
<i>1000.0</i>	<i>76.5</i>	<i>1285.7</i>	<i>5000.0</i>	
240,000,000	1,100,000	62,000,000		
<i>19,200.0</i>	<i>250.0</i>	<i>6888.8</i>		

The general trend was for organisms which grow best at 37° to reach a maximum during the earlier stages of the period of holding, while those growing best at 20° and at 0° continued to increase and reached their highest values during the later stages of the experiment.

If the ratio of increase in the raw untreated milk at each period of analysis and at each temperature of incubation for each group of organisms be compared with the similar ratio in the reinfected sterile milk, it is seen that, in 42 of the 46 cases, 91.3 per cent, the ratio is higher in the raw milk than in the reinfected milk. Therefore, at 0°C. the raw milk has been the more suitable medium for the organisms and has given rise to a greater rate of proliferation.

The relative rate of growth of milk organisms in raw and pasteurized milk has been studied by several investigators. According to St. John and Pennington<sup>32</sup> when raw clean milk and reinfected pasteurized clean milk of approximately the same bacterial content are held at the temperature of the room and of the ice box, the milk organisms proliferate more rapidly in the reinfected than in the raw sample. Rickards<sup>33</sup> likewise found that bacteria increase more rapidly in pasteurized than in unpasteurized milk at the temperature of the ice box. Ayers and Johnson<sup>34</sup> state that their results, obtained on milk kept at 10°C., "tend to prove that bacteria do increase approximately the same in pasteurized as in raw milk, provided their initial counts are practically alike."

The mode of preparing the reinfected sterile milk in the present research was the same procedure as used by St. John and Pennington in their study of reinfected pasteurized milk. The rate of bacterial growth was greater in the reinfected pasteurized milk than in the raw milk but was greater in the raw milk than in the reinfected sterile milk. On the other hand, in the experiments on cream described later in this paper, the rate of bacterial increase was greater in the reinfected sterile cream than in the raw cream. The following explanation is offered for these

<sup>32</sup> *Loc. cit.*

<sup>33</sup> Rickards: *Amer. Journ. of Public Hygiene*, xix (New Series, v), p. 507, 1909.

<sup>34</sup> Ayers and Johnson: U. S. Dept. of Agric., Bureau of Animal Industry, Bulletin 126, 1910, p. 52.

phenomena. The pasteurized milk underwent no marked change in color and suffered no chemical change save, possibly, a partial coagulation of the lactalbumin; pasteurization had been attained by holding the milk at 79°C. for 20 minutes. The recent work of Rupp<sup>35</sup> demonstrates that the only appreciable chemical change produced in milk by pasteurization is a partial coagulation of the lactalbumin. Thus Rupp found that pasteurization of milk by holding at 71.1°C. for 30 minutes coagulated 30.87 per cent of the total lactalbumin of the milk. This investigator also noted that an increase in the temperature of pasteurization, the time factor remaining constant, gave rise to an increased coagulation of lactalbumin.

The sterilized milk had a marked light golden color; the major portion of the lactalbumin had been coagulated; and a portion of the lactose must have undergone decomposition, for the lactose content should have risen to a slight extent on account of the concentration during sterilization, yet the sterilized milk contained less lactose than did the fresh raw milk. One evidence of this decomposition of lactose is the greater acidity of the sterile than of the fresh raw milk on the initial analysis. The increase in lactic acid, however, is not sufficient to account for the decrease in lactose, and it appears probable that certain other decomposition products of the lactose may exert an inhibitory action on the bacterial growth in the reinfected sterile milk.

In the cream, the change in color as the result of sterilization was not noted, and moreover the lactose content of cream would be much less than that of milk, so the inhibitory substances would be formed to but a slight degree, if at all.

During the process of sterilization, however, the complex molecules of the organic constituents of the milk may possibly be rearranged in a manner which escapes detection by the ordinary methods of milk analysis, and the value of the milk as a nutrient medium for the rather fastidious organisms may be lessened, thus accounting for the slow rate of proliferation in the reinfected sterile milk.

<sup>35</sup> Rupp: U. S. Dept. of Agric., Bureau of Animal Industry, Bulletin 166, 1913.

*The bacterial flora of the raw untreated milk and of the reinfected sterile milk.*

The organisms which were isolated in pure culture from each sample of milk at each period of analysis are enumerated in Table V, according to the temperature of incubation of the plate on which each was found. The predominant species of organism at each temperature of incubation and each period of analysis of each sample is designated by a double asterisk (\*\*), the species next numerous is designated by a single asterisk (\*); at times, two organisms shared the premier position, in such cases both are designated by a double asterisk, and the single asterisk is omitted.

In the fresh *raw, untreated* milk, on the plates incubated at 37°, the predominant species was *Micrococcus ovalis* (Escherich); throughout the period of holding this organism tended to retain its position, although it twice shared that position with *Micrococcus aurantiacus* (Cohn), and once was displaced by the latter. On the plates incubated at 20°, *M. aurantiacus* held the premier position most of the time throughout the experiment, although it was once equalled and twice exceeded by *M. ovalis*. On the plates incubated at 0°, *M. aurantiacus* predominated until the very last analysis when it gave place to *M. ovalis*. At all three temperatures, whenever *M. ovalis* predominated, *M. aurantiacus* held the second place with respect to frequency of occurrence, and *vice versa*. Both these micrococci are acid formers. Apparently, *M. aurantiacus* preferred the lower temperatures of incubation, while *M. ovalis* became acclimated to those temperatures during the period of holding at 0°C. This process of acclimatization was also undergone by other organisms, thus *Bacterium flexuosum* was not found on the plates sown with fresh milk and incubated at 0°, but occurred on the 0° plates beginning with 7 days of holding and continuing to the end of the experiment.

On the other hand, some organisms soon disappeared from the milk during holding. Thus *Micrococcus acidi lactici* was recovered on the plates incubated at 37° from both the fresh milk and the milk after storage for 7 days, but thereafter could not be isolated from such plates. This organism was never found on the plates incubated at 20° and at 0°.

In the fresh *reinfected, sterile* milk, the predominant organism at 37° was *Micrococcus ovalis* (Escherich), at 20° and at 0° *Micro-*

TABLE V.

*Organisms isolated in pure culture from raw untreated and reinfected sterile milk, kept at 0° C.*

PERIOD IN DAYS OF HOLDING MILK AT 0° C.	FROM THE PLATES INCUBATED AT °C.	RAW UNTREATED MILK	REINFECTED STERILE MILK
Fresh	37	M. acidilactici (Linder) M. albus liquifaciens *M. aurantiacus (Cohn) **M. ovalis (Escherich) S. flava	M. albus liquifaciens **M. ovalis (Escherich) S. farcinica S. flava *Penicillium
	20	M. albus liquifaciens **M. aurantiacus (Cohn) *M. ovalis (Escherich) B. flexuosum (Wright) S. flava	**M. aurantiacus (Cohn) *M. ovalis (Escherich) B. flexuosum (Wright) S. flava Penicillium
	0	**M. aurantiacus (Cohn) *M. ovalis (Escherich)	**M. aurantiacus (Cohn) *M. ovalis (Escherich)
7	37	M. acidilactici (Linder) M. albus liquifaciens *M. aurantiacus (Cohn) **M. ovalis (Escherich) B. flexuosum (Wright) B. detrudens (Wright)	M. albus liquifaciens *M. aurantiacus (Cohn) **M. ovalis (Escherich) B. flexuosum (Wright)
	20	**M. aurantiacus (Cohn) *M. ovalis (Escherich) B. flexuosum (Wright) S. flava	M. albus liquifaciens **M. aurantiacus (Cohn) *M. ovalis (Escherich) B. flexuosum (Wright)
	0	**M. aurantiacus (Cohn) *M. ovalis (Escherich) B. flexuosum (Wright) S. flava	*M. aurantiacus (Cohn) **M. ovalis (Escherich)
14	37	**M. aurantiacus (Cohn) **M. ovalis (Escherich) B. flexuosum (Wright)	M. aerius *M. aurantiacus (Cohn) **M. ovalis (Escherich)
	20	*M. aurantiacus (Cohn) **M. ovalis (Escherich) S. flava	*M. aurantiacus (Cohn) **M. ovalis (Escherich) B. flexuosum (Wright) S. flava

\*\* The predominant species of organism at each temperature of incubation and at each period of analysis.

\* The second most numerous species of organism at each temperature of incubation and at each period of analysis.

TABLE V.—Continued.

PERIOD IN DAYS OF HOLDING MILK AT 0°C.	FROM THE PLATES INCUBATED AT °C.	RAW UNTREATED MILK	REINFECTED STERILE MILK
14	0	**M. aurantiacus (Cohn) *M. ovalis (Escherich) B. flexuosum (Wright) S. flava	**M. aurantiacus (Cohn) *M. ovalis (Escherich) B. flexuosum (Wright) S. flava
	37	**M. aurantiacus (Cohn) *M. ovalis (Escherich) B. flexuosum (Wright) S. flava	**M. aurantiacus (Cohn) *M. ovalis (Escherich)
21	20	**M. aurantiacus (Cohn) *M. ovalis (Escherich) B. flexuosum (Wright) S. flava	M. albus liquifaciens **M. aurantiacus (Cohn) *M. ovalis (Escherich) B. flexuosum (Wright) S. flava
	0	**M. aurantiacus (Cohn) *M. ovalis (Escherich) B. flexuosum (Wright)	**M. aurantiacus (Cohn) *M. ovalis (Escherich) B. flexuosum (Wright)
28	37	**M. aurantiacus (Cohn) *M. ovalis (Escherich)	**M. aurantiacus (Cohn) *M. ovalis (Escherich)
	20	*M. aurantiacus (Cohn) **M. ovalis (Escherich) S. flava	M. aurantiacus (Cohn) **M. ovalis (Escherich) *B. flexuosum (Wright)
	0	*M. aurantiacus (Cohn) *M. ovalis (Escherich) B. flexuosum (Wright)	*M. aurantiacus (Cohn) **M. ovalis (Escherich) B. flexuosum (Wright)
	37	*M. aurantiacus (Cohn) **M. ovalis (Escherich) S. flava	**M. aurantiacus (Cohn) *M. ovalis (Escherich) *S. flava
	20	**M. aurantiacus (Cohn) *M. ovalis (Escherich) B. flexuosum (Wright) S. flava	**M. aurantiacus (Cohn) *M. ovalis (Escherich) B. flexuosum (Wright) S. flava
35	0	*M. aurantiacus (Cohn) **M. ovalis (Escherich) B. flexuosum (Wright)	**M. aurantiacus (Cohn) *M. ovalis (Escherich)

\*\* The predominant species of organism at each temperature of incubation and at each period of analysis.

\* The second most numerous species of organism at each temperature of incubation and at each period of analysis.

*coccus aurantiacus* (Cohn). Throughout the period of holding, at each of the three temperatures of incubation, *M. ovalis* predominated twice, *M. aurantiacus* thrice. The two lower temperatures, at the beginning of the experiment offered a more favorable environment to *M. aurantiacus* than to *M. ovalis*, yet the latter exhibited a tendency to become acclimated during holding of the milk at 0°, as was shown by its predominance at 28 days on the plates incubated at 20° and at 0°. These two acid-forming micrococci—*M. aurantiacus* and *M. ovalis*—almost invariably were the two more numerous species. On the plates at 37°, however, the second place in the fresh sample was occupied by *Penicillium*, and in the final sample was shared by *M. ovalis* and *Streptothrix flava*, while on the plates at 20° the second place at 28 days was occupied by *Bacterium flexuosum*. The tendency to become acclimated to the low temperature was also shown by *Bacterium flexuosum* which disappeared from the 37° plates after the first week of holding, appeared on the 20° plates beginning with the first week and on the 0° plates beginning with the second week. The opposite tendency—to disappear entirely from the milk during holding—was exhibited by *Streptothrix farcinica* which was found only on the 37° plates of the fresh sample; it failed to develop on the 20° and 0° plates of that sample and was never found during the subsequent analyses.

#### THE BACTERIAL CHANGES IN THE CREAM DURING HOLDING.

In the raw, untreated cream, on the plates incubated at 37° the total count per cc. increased progressively up to 14 days, then decreased; the anaerobes and facultatives rose during the first week, then decreased; the acid formers underwent a progressive increase for 14 days, then decreased. On the plates incubated at 20°, the total count per cc. increased irregularly to a maximum at 21 days, followed by a decrease on the final analysis; the anaerobes and facultatives attained their highest value at 7 days, then exhibited a marked tendency to decrease progressively; the acid formers reached their highest values at 7 and 21 days, there being a trend toward a maximum during the middle of the experiment, then a decrease toward the close of the experiment; the liquefiers increased more or less regularly throughout the entire experiment. On the

TABLE VI. *Bacterial count*  
(The figures in Roman represent bacteria)

PERIOD OF KEEPING IN DAYS	PLATES INCUBATED AT °C	RAW UNTREATED CREAM			
		Total count per cc.	Anaerobes and Facultatives per cc.	Acid Formers per cc.	Liquefiers per cc.
Fresh.....	37	5,900,000 1	1,400,000 1	550,000 1	
	20	6,100,000 1	1,700,000 1	1,800,000 1	320,000 1
	0	2,200,000 1	56,000 1	1,100,000 1	
7.....	37	15,000,000 2.5	5,000,000 3.6	10,000,000 18.2	
	20	97,000,000 15.9	29,000,000 17.1	72,000,000 40.0	7,000,000 21.9
	0	23,000,000 10.5	4,000,000 71.4	5,000,000 4.5	
14.....	37	58,000,000 9.8	3,300,000 2.4	20,000,000 36.4	
	20	53,000,000 8.7	8,000,000 4.7	47,000,000 26.1	3,000,000 9.4
	0	12,000,000 5.5	1,200,000 21.4	5,000,000 4.5	
21.....	37	320,000 0.05		50,000 0.09	
	20	120,000,000 19.7	290,000 0.17	70,000,000 38.9	14,000,000 43.8
	0	120,000,000 54.5	12,000,000 214.3	26,000,000 23.6	
28.....	37	420,000 0.07	130,000 0.09	180,000 0.33	
	20	10,000,000 1.6	1,000,000 0.59	3,600,000 2.0	19,000,000 59.4
	0				

plates incubated at 0°, the *total count per cc.*, *anaerobes and facultatives*, and *acid formers* all showed a distinct tendency to increase progressively during the entire period of holding.

In the *reinfected, sterile* cream, on the plates incubated at 37°, the *total count per cc.*, *anaerobes and facultatives* and *acid formers* rose progressively up to 14 days, then decreased on the final analy-



*in cream kept at 0°C.*

*in Italic represent ratios of increase.)*

REINFECTED STERILE CREAM				FORMOLIZED RAW CREAM
Total count per cc.	Anaerobes and Facultatives per cc.	Acid Formers per cc.	Liquefiers per cc.	Total count per cc.
850,000	15,000	38,000		160,000
<i>1</i>	<i>1</i>	<i>1</i>		
1,300,000	650,000	750,000	33,500	0
<i>1</i>	<i>1</i>	<i>1</i>	<i>1</i>	
13,000	2,600	5,600		0
<i>1</i>	<i>1</i>	<i>1</i>		
11,000,000	600,000	8,600,000		0
<i>12.9</i>	<i>40.0</i>	<i>226.3</i>		
48,000,000	9,000,000	28,000,000	15,000,000	0
<i>36.9</i>	<i>13.8</i>	<i>34.7</i>	<i>447.8</i>	
20,000,000		8,400,000		0
<i>1538.5</i>		<i>1500.0</i>		
19,000,000	4,000,000	9,000,000		0
<i>22.4</i>	<i>266.7</i>	<i>236.8</i>		
38,000,000	16,000,000	35,000,000	2,000,000	
<i>29.2</i>	<i>24.6</i>	<i>46.7</i>	<i>59.7</i>	
7,800,000	1,000,000	3,700,000		
<i>600.0</i>	<i>384.6</i>	<i>660.7</i>		
40,000		20,000		3
<i>0.05</i>		<i>0.53</i>		
130,000,000	160,000	56,000,000	28,000,000	
<i>100.0</i>	<i>0.25</i>	<i>74.7</i>	<i>835.8</i>	
120,000,000	1,500,000	30,000,000		
<i>9230.8</i>	<i>576.9</i>	<i>5357.1</i>		1

sis. On the plates incubated at 20°, the *total count per cc.*, *acid formers* and *liquefiers* rose more or less progressively to their highest values on the concluding day of the experiment; the *anaerobes and facultatives* rose progressively during the first fortnight of holding, then suffered an enormous decrease during the third and last week in storage. On the plates incubated at 0°, the *total count per cc.*,

*anaerobes and facultatives* and *acid formers* rose with more or less regularity to maximum values on the twenty-first and concluding day of the experiment.

In the fresh *formolized*, raw cream, organisms were present, but subsequent analyses demonstrated that the cream had become sterile.

The *sterile* cream retained its sterility throughout the period of holding, as was demonstrated by platings made on the initial and final analyses.

Since the initial counts for all groups of organisms differ widely in the raw untreated and reinfected sterile cream, comparison of the counts themselves at different periods of the experiment cannot be made. A comparison of the ratio of increase of the various groups of organisms in the two kinds of cream, however, will be made during the discussion of those ratios (see page 363).

As a rule, the optimum temperature of incubation for all groups of bacteria remained at 20° throughout the entire period of study.

#### *Ratio of increase of the organisms.*

The maximum count obtained during the period of holding with each group of organisms at each temperature of incubation is always compared with the count obtained with that group of organisms at the same temperature of incubation at the beginning of the experiment, using the latter figure as unity in the ratio.

RAW, UNTREATED CREAM. *Total count per cc.* At 37° the maximum ratio of increase was attained at 14 days and was 9.8 times the count on the fresh cream. At 21 days the maximum ratio at 20° (1:19.7) and at 0° (1:54.5) was reached. *Anaerobes and facultatives.* The maximum increase at 37° (1:3.6) and at 20° (1:17.1) occurred at 7 days, while the maximum for 0° (1:214.3) was attained at 21 days. *Acid formers.* At 37° the maximum was reached at 14 days with the ratio 1:36.4, while at 0° the maximum was at 21 days with the ratio 1:21.7; the maximum ratio at 20° was 1:40.0 at 7 days, and was closely followed by the ratio 1:38.9 at 21 days. *Liquefiers* reached their highest value at 28 days with a ratio of increase 1:59.4.

REINFECTED, STERILE CREAM. *Total count per cc.* The maximum increase at 37° (1:22.4) occurred at 14 days, the maximum at 20° (1:100.0) and at 0° (1:9230.8) took place at 21 days. *Anaerobes and facultatives.* The maximum ratio of increase at 37° (1:266.7) and at 20° (1:24.6) was attained at 14 days, while the maximum at 0° (1:567.9) was at 21 days. *Acid formers.* At 37° the greatest increase (1:236.8) was attained at 14 days, while the maximum increase at 20° (1:74.7) and at 0° (1:5371.1) was found at 21 days. The greatest ratio of increase for liquefiers was 1:835.8 at 21 days.

The organisms which proliferate best at 37° always reached their highest numbers by the fourteenth day of holding, while the maximum ratio of increase of the organisms which grow best at 20° and at 0° almost invariably occurred on the twenty-first day.

Upon comparison of the ratio of increase in the raw untreated cream at each period of analysis and at each temperature of incubation for each group of organisms with the similar ratio in the reinfected sterile cream, it is seen that, in 25 of the 27 cases, 92.6 per cent, the ratio is higher in the reinfected cream than in the raw cream. Therefore, at 0°C., the reinfected cream has been a more suitable medium for bacterial reproduction and has given rise to a greater rate of growth.

*The bacterial flora of the raw untreated cream and of the reinfected sterile cream.*

The mode of tabulation of the organisms is the same as was used for the bacterial flora of the milk. In the fresh raw, untreated cream, on the plates incubated at 37° and at 20°, *Micrococcus aurantiacus* (Cohn) predominated, while on the plates incubated at 0° *Micrococcus ovalis* (Escherich) occupied that position. During the period of holding, on the plates at 37°, *M. ovalis* predominated until the last analysis when it gave place to *M. aurantiacus*; on the plates at 20°, *M. ovalis* occupied the first position but was displaced by *M. aurantiacus* during the last two weeks of the experiment; on the plates at 0°, *M. aurantiacus* was the predominant organism throughout the entire period of holding. The first and second positions were almost invariably held by the two acid-forming micrococci, *M. aurantiacus* and *M. ovalis*; the only exception occurred at 14 days on the plates incubated at 20° where *Bacterium aerophilum* occupied second place. On the whole the storage at a temperature of 0°C. exerted a favorable influence on *M. aurantiacus* and an unfavorable influence on *M. ovalis*, for the former gradually displaced the latter even on the plates grown at 37°. The disappearance of certain organisms during holding was illustrated by *M. acidi lactis* and *Bacillus detrudens*, which were isolated from plates sown with fresh cream and incubated at 37°; these organisms, however, were never found in the cream during the period of holding.

TABLE VII.

*Organisms isolated in pure culture from raw untreated and reinfected steril cream, kept at 0°C.*

PERIOD IN DAYS OF HOLDING CREAM AT 0°C.	FROM THE PLATES INCUBATED AT °C.	RAW UNTREATED CREAM	REINFECTED STERILE CREAM
Fresh	37	M. acidilactis **M. aurantiacus (Cohn) *M. ovalis (Escherich) B. flexuosum (Wright) B. detrudens (Wright) S. Rosenbachii (Kruse)	**M. aurantiacus (Cohn) *M. ovalis (Escherich) B. detrudens (Wright) S. Rosenbachii (Kruse)
	20	M. acidilactici **M. aurantiacus (Cohn) *M. ovalis (Escherich) B. aerophilum B. flexuosum (Wright) S. flava	*M. aurantiacus (Cohn) **M. ovalis (Escherich) B. aerophilum B. flexuosum (Wright) S. farcinica S. Rosenbachii (Kruse)
	0	*M. aurantiacus (Cohn) **M. ovalis (Escherich) B. aerophilum B. flexuosum (Wright)	**M. aurantiacus (Cohn) *M. ovalis (Escherich) B. aerophilum B. flexuosum (Wright)
7	37	M. alvi *M. aurantiacus (Cohn) **M. ovalis (Escherich) S. Rosenbachii (Kruse)	M. acidilactici M. alvi *M. aurantiacus (Cohn) **M. ovalis (Escherich) S. Rosenbachii (Kruse)
	20	*M. aurantiacus (Cohn) **M. ovalis (Escherich) B. flexuosum (Wright) S. flava	M. aurantiacus (Cohn) **M. ovalis (Escherich) *S. flava
	0	**M. aurantiacus (Cohn) *M. ovalis (Escherich) B. aerophilum S. flava	**M. aurantiacus (Cohn) *M. ovalis (Escherich) B. aerophilum

\*\* The predominant species of organism at each temperature of incubation and at each period of analysis.

\* The second most numerous species of organism at each temperature of incubation and at each period of analysis.

TABLE VII—Continued.

PERIOD IN DAYS OF HOLDING CREAM AT 0°C.	FROM THE PLATES INCUBATED AT °C.	RAW UNTREATED CREAM	REINFECTED STERILE CREAM
14	37	*M. aurantiacus (Cohn) **M. ovalis (Escherich) B. aerophilum B. flexuosum (Wright)	**M. aurantiacus (Cohn) *M. ovalis (Escherich) B. aerophilum B. flexuosum (Wright) S. flava
		*M. aurantiacus (Cohn) **M. ovalis (Escherich) B. aerophilum B. flexuosum (Wright) S. flava	*M. aurantiacus (Cohn) **M. ovalis (Escherich) B. aerophilum S. flava S. Rosenbachii (Kruse)
		**M. aurantiacus (Cohn) *M. ovalis (Escherich) B. aerophilum S. flava	**M. aurantiacus (Cohn) *M. ovalis (Escherich) B. aerophilum
	21	*M. aurantiacus (Cohn) **M. ovalis (Escherich) B. aerophilum	M. acidi lactici **M. aurantiacus (Cohn) M. ovalis (Escherich) *B. aerophilum S. Rosenbachii (Kruse)
		**M. aurantiacus (Cohn) *M. ovalis (Escherich) B. aerophilum	**M. aurantiacus (Cohn) *M. ovalis (Escherich) B. aerophilum B. flexuosum (Wright) S. flava
		**M. aurantiacus (Cohn) *M. ovalis (Escherich) B. aerophilum B. flexuosum (Wright)	**M. aurantiacus (Cohn) *M. ovalis (Escherich) B. aerophilum B. flexuosum (Wright)
28	37	**M. aurantiacus (Cohn) *M. ovalis (Escherich) B. aerophilum	
	20	**M. aurantiacus (Cohn) *M. ovalis (Escherich) B. aerophilum	

\*\* The predominant species of organism at each temperature of incubation and at each period of analysis.

\* The second most numerous species of organism at each temperature of incubation and at each period of analysis.

In the *reinfected, sterile* cream, on the plates at 37°, *Micrococcus aurantiacus* (Cohn) predominated in the fresh sample and held that position throughout the entire experiment save at 7 days, where it gave place to *Micrococcus ovalis* (Escherich). On the plates at 20°, *M. ovalis* was the predominating species save on the final analysis when it was displaced by *M. aurantiacus*. On the plates at 0°, *M. aurantiacus* held the first position throughout the entire period of holding. At all three temperatures of incubation, the first and second positions were usually shared by *M. ovalis* and *M. aurantiacus*; however, the second position at 21 days on the plates at 37° was held by *Bacterium aerophilum*, and at 7 days on the plates at 20° by *Streptothrix flava*. The trend for some organisms to disappear during holding was illustrated by *Bacillus destrudens*, which was isolated from the plates sown with the fresh sample and incubated at 37°; at subsequent periods of analysis, the organism was never found.

#### GENERAL CONCLUSIONS.

In milk and cream which are held at a temperature of 0°C., the following phenomena are noted:

The proteolysis of the casein is, primarily, of bacterial origin.

The proteolysis of the lactalbumin is due, primarily, to native enzymes of the milk.

The bacterial flora and the native milk enzymes by their combined action give rise to more rapid proteolytic changes than are produced by either agent alone.

The general trend of the proteolysis by bacteria, by enzymes, and by the combined action of these two agents, involves a breaking down of the true proteins and their passage through caseose and peptone to amino-acids.

The fermentation of the lactose with the formation of lactic acid is largely, if not exclusively, due to bacterial action.

The digestion of the protein, the fermentation of the lactose and the increase in acidity are progressive changes, and are accompanied by more or less progressive lowerings of the freezing point of the milk.

The depression of the freezing point of the cream is to be ascribed to chemical changes in its protein and lactose.

The lecithin of the cream was not decomposed during the period of holding.

The iodine number and the index of refraction of the butter-fat remained unchanged, while the Reichert-Meissl number underwent no marked increase or decrease in any of the samples.

The hydrolysis of the fat and the increase in acid value, which is usually progressive, are due to the action of bacteria.

The Hehner number always becomes greater; the saponification number usually increases, although it underwent progressive decreases in the reinfected sterile cream. The increase in Hehner number, accompanied by a decrease in saponification number, in the reinfected sterile cream is to be ascribed to bacterial action. The simultaneous increase in the two constants in the sterilized cream is doubtless due to oxidation and the fine state of division of the butter-fat in the cream, possibly aided by a thermostable inorganic catalyst; these same causes, plus the action of the native enzymes, must give rise to the simultaneous increase in the Hehner and saponification numbers of the formolized raw cream; while the simultaneous rise in these two constants in the raw untreated cream are the resultant of the action of bacteria, native enzymes, oxidation, inorganic catalysts and fine state of division of the butter-fat.

The simple reductases and the trikresol oxidases of the *milk* may be native enzymes and may also be of bacterial origin, while the aldehyde reductase and the guaiac oxidases, apparently, are of bacterial origin.

Both varieties of reductase and the trikresol oxidases are native enzymes of the *cream* and are also secreted by the microorganisms, while the guaiac oxidases probably have their origin in bacterial action.

The catalase of both the milk and the cream is a native enzyme and is also due to the activity of microorganisms.

The two varieties of oxidase, the two varieties of reductase and the catalase retain their activity in spite of the prolonged exposure to a temperature of 0°C.

During the holding at 0°C., the organisms of the raw untreated and reinfected sterile milk and cream undergo an increase, which is most striking in the raw untreated milk.

In both milk and cream, the organisms which proliferate best at 37°C. reach a maximum growth during the earlier stages of the period of holding, while those growing best at 20°C. and at 0°C. continue to increase and attain their highest values during the later stages of the experiment.

Some organisms disappear during holding, others become acclimated to the lower temperatures of incubation.

The total count per cc. at 37° was practically the same in the raw untreated and reinfected sterile milk; in over 90 per cent of the subsequent determinations the rate of increase of the various groups of organisms was greater in the raw than in the reinfected sample.

In over 90 per cent of the determinations made during the period of holding, the rate of increase of the various groups of organisms was greater in the reinfected sterile cream than in the raw untreated cream.

Almost invariably *Micrococcus aurantiacus* (Cohn), and *Micrococcus ovalis* (Escherich), which belong to the group of acid-formers, were the predominant organisms of both raw untreated and reinfected sterile milk and cream.



# THE REACTION OF SOME PURINE, PYRIMIDINE, AND HYDANTOIN DERIVATIVES WITH THE URIC ACID AND PHENOL REAGENTS OF FOLIN AND DENIS.

BY HOWARD B. LEWIS AND BEN H. NICOLET.

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(Received for publication, October 10, 1913.)

In the course of an investigation by one of us<sup>1</sup> on the behavior of certain thioderivatives of hydantoin in the animal organism, it was observed that the substances studied reacted with the reagents of Folin and Denis<sup>2</sup> with the development of the blue color described as typical for uric acid. The possibility of using this reaction for the detection of the compounds under investigation at once suggested itself, and an examination of various related substances in regard to their behavior toward the new reagents was undertaken. Recently Funk and Macallum<sup>3</sup> have studied the reaction with certain purines, pyrimidines, and related substances of biochemical importance. Inasmuch as our results supplement and extend the work of these investigators, we offer them in the hope that they may prove of value in pointing out a line of attack for the solution of the problem of the chemical basis of the reactions. For many of the compounds studied we are indebted to Profs. Lafayette B. Mendel and T. B. Johnson, who have placed them at our disposal.

With the exception of thiourea no substance was observed to react typically with the phenol reagent, which does not contain a phenol group or react with the uric acid reagent. *N*-*i*-methyl-tyrosine reacts positively with the phenol reagent, but the related

<sup>1</sup> Lewis: this *Journal*, xiv, pp. 245-56, 1913.

<sup>2</sup> Folin and Denis: this *Journal*, xii, p. 239, 1912.

<sup>3</sup> Funk and Macallum: *Biochem. Journ.*, vii, pp. 356-58, 1913.

TABLE I.

	URIC ACID REAGENT	PHENOL REAGENT
1. Hydantoin.....	—	—
2. Hydantoin-4-acetic acid.....	—	—
3. Hydantoin-4-propionic acid.....	—	—
4. <i>p</i> -Hydroxybenzylhydantoin.....	—	+++
5. <i>p</i> -Aminobenzylhydantoin.....	—	—
6. Parabanic acid.....	—	—
7. Aminocarboxyhydantoin.....	+++	+++
8. 2-Thiohydantoin.....	+++	+++
9. 2-Thio-4-methylhydantoin.....	+++	+++
10. 2-Thiohydantoin-4-acetic acid.....	+++	+++
11. 2-Thio-4-benzalhydantoin.....	—	—
12. 2-Thio-4-benzylhydantoin.....	+++	+++
13. 1-Phenyl-2-thio-4-anisalhydantoin.....	—	—
14. 1-Phenyl-2-thio-4-anisylhydantoin.....	+++	+++
15. 1-Phenyl-2-thio-4-cinnamalhydantoin.....	—	—
16. 1-Phenyl-2-thio-4-furfuralhydantoin.....	—	—
17. 1-Phenyl-2-thiohydantoin.....	+++	+++
18. 3-Phenyl-2-thiohydantoin.....	+++	+++
19. 1,3-Diphenyl-2-thiohydantoin.....	+++	+++
20. 1-Phenyl-2-thio-4-piperonalhydantoin.....	—	—
<i>Purine derivatives.</i>		
21. 2,8-Dioxypurine.....	—	—
22. 2,8-Dioxy-6-methylpurine.....	—	—
23. 2,8-Dioxy-6,9-dimethylpurine.....	—	—
24. 2,8-Dioxy-9-methylpurine.....	—	—
25. 2,8-Dioxy-1,9-dimethylpurine.....	—	—
26. 2-Oxy-9-methylpurine.....	—	—
27. 2-Oxy-6,9-dimethylpurine.....	—	—
28. 2-Oxy-6,8,9-trimethylpurine.....	—	—
29. 2,6-Dioxy-8-methylpurine.....	—	—
30. 2,6-Dioxy-1,7-dimethylpurine.....	—	—
31. 2,6-Dioxy-1,3-dimethylpurine.....	—	—
32. 2,6-Dioxy-1,3,7-trimethylpurine.....	—	—
33. Sodium uroxanate.....	—	—
34. 2-Thio-6-oxypurine.....	+++	+++
35. 2,8-Dithio-6-oxypurine.....	+++	+++
36. 2-Thio-6,8-dioxypurine.....	+++	+++
37. 2-Thioglycollic acid-6-oxypurine.....	—	—
38. 2,8-Dithioglycollic acid-6-oxypurine.....	—	+
39. 2-Thioglycollic acid-6,8-dioxypurine.....	±	+

TABLE I.—Continued.  
*Pyrimidine derivatives.*

	URIC ACID REAGENT	PHENOL REAGENT
40. Cytosine.....	—	
41. Isocytosine.....	—	
42. 6-Methylcytosine.....	—	
43. Thymine.....	—	
44. 1-Methylthymine.....	—	
45. 1,3-Dimethylthymine.....	—	
46. Diethylbarbituric acid (Veronal).....	—	
47. Barbituric acid.....	—	
48. Violuric acid.....	—	
49. 2-Thiovioluric acid.....	+++	+++
50. Cyanacetylguanidine.....	—	
51. 2-Phenylamino-6-oxypyrimidine.....	—	
52. 2-Ethylmercaptopyrimidine.....	—	
53. 2,4-Diamino-6-oxypyrimidine sulphate.....	—	
54. 2,4,5-Triamino-6-oxypyrimidine.....	+++	
55. Malonylguanidine.....	—	
56. 5-Aminomalonylguanidine hydrochloride.....	+++	
57. 2-Thiopseudouric acid.....	+++	+++
58. 2-Thiouramil.....	+++	+++

*Miscellaneous compounds.*

59. Thiotyrosine.....	+++	+++
60. Thiourea.....	—	+++
61. Thiobenzamide.....	+	+
62. N-methyldiiodtyrosine.....	—	+++
63. N- <i>i</i> -methyltyrosine.....	—	+++
64. $\alpha$ -Methylamino- $\beta$ - <i>p</i> -methoxyphenylpropionic acid.....	—	—
65. Glycollic acid.....	—	—
66. Allantoin.....	—	—
67. Malonaminourethane.....	+++	+++
68. Dithiodimethylpiperazine.....	+++	++
69. Benzoylthiourea.....	—	—
70. Benzoylphenylthiourea.....	—	—

$\alpha$ -methylamin oparamethoxyphenylpropionic acid in which a methyl group has been substituted for the hydrogen of the hydroxyl group fails to give the reaction. N-methyldiiodtyrosine, which does not respond to Millon's test, gives a positive reaction with the

phenol reagent, indicating that it is not a necessary condition for this reaction as for Millon's that the position ortho to the hydroxyl group in the benzene ring shall be unsubstituted. Thiotyrosine reacts positively with the phenol reagent, but inasmuch as it also reacts positively with the uric acid reagent and as will be discussed later sulphur seems to play a rôle here, no conclusion as to the effect of -SH groups replacing -OH groups can be drawn. Of the cyclic compounds examined which contained no sulphur, only three, 4-aminocarboxyhydantoin, 2,4,5-triamino-6-oxypyrimidine, and 5-aminomalonylguanidine react positively with the uric acid reagent. The striking feature possessed by these three compounds in common, in contrast with the closely related hydantoin, 2,4-diamino-6-oxypyrimidine, and malonylguanidine, none of which gives a positive reaction, is the presence of an amino group. In 2,4,5-triamino-6-oxypyrimidine and 5-malonylguanidine, the amino group which determines the reaction is in the 5-position as is evidenced by the inactivity of the 2,4-diamino compound and malonylguanidine respectively. It is interesting to note that malonaminourethane of which 4-aminocarboxyhydantoin is the cyclic anhydride is the only acyclic compound free from sulphur which reacted positively with the uric acid reagent. The activity of amino groups is in harmony with the observations of Folin and Denis and Funk and Macallum, that monohydric phenols do not react with the uric acid reagent unless an amino group is present in the benzene ring (*e. g.*, 2- or 3-amino-tyrosine).

With the exception of the thiopurines, none of the purines studied react positively. Neither xanthine nor its isomer, nor any of their methyl derivatives react. Allantoin and uroxic acid, both oxidation products of uric acid, fail to react.

The presence of sulphur replacing oxygen in the 2-position of a purine, pyrimidine, or hydantoin, gives rise to a positive reaction with both reagents (*cf.* 8-20, 34-36, 49, 57, 58). In the case of the condensation products of thiohydantoins with aldehydes, derivatives in which the carbon in the 4-position is unsaturated (11, 13, 15, 16, 20) the reaction is negative. When reduced the resulting products react positively (*cf.* 11 and 12, 13 and 14). That sulphur replacing oxygen rather than mercapto sulphur is a condition for a positive test is shown by the negative reactions

of the mercapto derivatives of the purines and pyrimidines (37-39, 52). Dithiodimethylpiperazine, the anhydride of the thiopoly-peptide, thioalanyl-thioalanine, reacts positively. Although thio-urea gives the test with the phenol reagent, neither of the two substituted thioureas (69, 70) gives a positive reaction.

The suggestion made by Funk and Macallum that in the purines the substitution of the hydrogen atoms of the ring lessens or destroys the power to react with the uric acid reagent, does not hold for the hydantoin ring. Those thiohydantoins in which substitution has occurred on the 1- or 3-carbon atom or on both (17-19), react as readily with the reagents as do the unsubstituted compounds.



## THE FORMATION OF GLUCOSE FROM PROPIONIC ACID IN DIABETES MELLITUS.

By ISIDOR GREENWALD.

(From the Chemical Laboratory of the Montefiore Home, New York.)

(Received for publication, October 15, 1913.)

It has been shown by Ringer<sup>1</sup> that the administration of propionic acid to phlorhizinized dogs is followed by the elimination of "extra glucose" equal in amount to that capable of being formed from the propionic acid if all three carbon atoms are used in the formation of glucose.

Shortly after the publication of this work we had under observation a patient who exhibited a G : N ratio of about 3.5. The case seemed to be eminently suitable for the comparison of glucose formation in phlorhizin glycosuria and in diabetes mellitus. Propionic acid being readily obtainable it seemed desirable to ascertain if it would give rise to glucose. This was found to be the case. The experiment was subsequently repeated upon the same patient and also upon two others with less severe forms of diabetes. In one patient, who had only a slight diabetes, the administration of propionic acid did not increase the excretion of glucose. Otherwise, a distinct rise was observed in every experiment. The excretion of acetone and  $\beta$ -hydroxybutyric acid was followed in some of the experiments. In only one of these was there an increase in the amount of these substances eliminated and in this case there was a further rise on the following day. Four typical experiments are summarized in the accompanying tables.

The propionic acid was prepared by Kahlbaum. The analytical methods employed were the Kjeldahl for nitrogen, the Benedict for glucose and the Shaffer for acetone and  $\beta$ -hydroxybutyric acid. The figures for the carbohydrate in the diet were calculated from the data given in Bulletin 28, U. S. Department of Agriculture.

<sup>1</sup> Ringer: *This Journal*, xii, p. 511, 1912.

# 376      Glucose Formation from Propionic Acid

As is evident from the tables, in the first experiment the "extra glucose" was almost exactly equivalent in amount to the propionic acid ingested. Later when the G:N ratio was lower, indicating an increased capacity for the oxidation of carbohydrates, the amount of glucose formed from the propionic acid administered was much diminished. The second patient, whose utilization of carbohydrates was much greater, excreted much less glucose as a result of the ingestion of propionic acid.

TABLE I.  
*Patient J. L.*

DATE	CARBO- HYDRATE IN FOOD	NITROGEN	GLUCOSE		G:N	"EXTRA GLUCOSE"	ACETONE	$\beta$ -HYDROXY- BUTYRIC ACID	
			Gross	Net					
	grams	grams	grams	grams		grams	grams	grams	
Jan.									
19	15.0	12.74	62.7	47.0	3.75				
20	13.1	13.88	62.8	29.7	3.56		4.16*	21.20*	
21	19.6	19.33	91.1	71.5	3.70		4.16*	21.20*	
22	16.0	17.07	99.1	83.1	4.86	20.8	4.00*	22.21*	20.0 gms. pro- pionic acid (equivalent to 24.3 gms. glucose.)
23	15.6	14.61	104.1	88.5	6.06	25.2	4.00*	22.21*	20.0 gms. pro- pionic acid (equivalent to 24.3 gms. glucose.)

The experiment was discontinued because the patient ate other food.

July									
20	17.2	6.93	34.8	17.6	2.53				
21	22.9	8.74	44.5	21.6	2.47				
22	19.6	10.37	56.7	27.1	3.58				
23	17.4	9.23	43.8	26.4	2.86		1.10	7.62	
24	13.0	7.72	58.8	45.8	5.92	23.9	1.43	11.41	36.65 grams propionic acid (equiv- alent to 44.57 grams glucose).
25	19.0	9.85	45.3	26.3	2.69		2.19	14.44	

\* Analyses made of two-day composites.



TABLE II.  
*Patient G. E.*

DATE	CARBO- HYDRATE IN FOOD	NITROGEN	GLUCOSE	
	<i>grams</i>	<i>grams</i>	<i>grams</i>	
June				
28	20.5	9.94	26.5	
29	19.3	11.52	26.5	
30	16.1	7.76	11.6	
July				
1	21.0	10.66	38.5	33.6 gms. propionic acid (equivalent to 39.9 gms. glucose).
2	25.5	7.15	18.9	
13	24.2	10.97	27.3	
14	25.2	9.92	26.9	
15	23.9	11.60	28.6	
16	24.9	10.26	37.3	56.8 gms. propionic acid (equivalent to 69.1 gms. glucose).
17	10.2	5.14	27.0	



## THE ACTION OF RADIUM EMANATION ON LIPASE.

By E. K. MARSHALL, JR. AND L. G. ROWNTREE.

(From the Laboratories of Physiological Chemistry and Pharmacology of the Johns Hopkins University.)

(Received for publication, October 17, 1913.)

It is claimed that both radium rays and radium emanation possess the power of activating certain enzymes. A certain accelerating influence has been shown for pepsin by the emanation (Bergell and Bickel<sup>1</sup>), for autolytic ferments by radium rays (Neuberg,<sup>2</sup> Wohlgemuth<sup>3</sup>), and by the emanation (Löwenthal and Edelstein<sup>4</sup>), for tyrosinase by the radium rays (Willcock<sup>5</sup>), for diastase from various origins (Löwenthal and Wohlgemuth<sup>6</sup>), and the uric acid forming ferments of the spleen by the emanation (Schultz<sup>7</sup>). On the other hand, a slight inhibitory effect was observed by Schmidt-Nielsen<sup>8</sup> for rennin by exposure to strong radium preparations. Trypsin, invertase, and emulsin are reported by Henri and Mayer<sup>9</sup> to be inactivated through long exposure to the rays.

The possibility of the increased rate of growth in plants<sup>10</sup> (germinating oats, for instance) being due in part to enzymatic activation has been suggested. The idea has been entertained that the therapeutic effect of radium treatment in gout is dependent on this cause. On account of the ease and accuracy of determining quantitatively lipolytic activity, the effect of the radium emanation in this connection has been investigated.

<sup>1</sup> *Verhandl. der Kongr. für inn. Med.*, Wiesbaden, 22d Kongress, 1905, p. 157.

<sup>2</sup> *Verhandl. d. deutsch. path. Gesellsch.*, vii, p. 157, 1904.

<sup>3</sup> *Ibid.*, vii, p. 158, 1904.

<sup>4</sup> *Biochem. Zeitschr.*, xiv, p. 484, 1908.

<sup>5</sup> *Journ. of Physiol.*, xxxiv, p. 207, 1906.

<sup>6</sup> *Biochem. Zeitschr.*, xxi, p. 476, 1909.

<sup>7</sup> *Ibid.*, xlviii, p. 86, 1913.

<sup>8</sup> *Mitt. a. Finsen's Med. Lysin. in Kopenh.*, Jena, 1906, 10 Heft, p. 107.

<sup>9</sup> *Compt. rend. de l'Acad. des Sci.*, cxl, p. 521, 1904.

<sup>10</sup> Falta and Schwartz: *Berl. klin. Wochenschr.*, xlviii, p. 605, 1911.

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The experiments have been carried out with the lipase of pig's liver and the lipase of the castor oil bean. In the case of liver, a 10 per cent "brei" was made, allowed to digest over night, and portions of the clear filtrate used in the experiments either diluted or undiluted. Different liver preparations were used. With the castor bean lipase, the beans were finely ground, extracted with ether in a Soxhlet and weighed portions of this powder used. In some of the experiments with the liver lipase, a saturated aqueous solution of ethyl butyrate was used as substrate, in all other cases 0.5 cc. of ethyl butyrate and 25 cc. of water as the solvent have been used. The mixtures were allowed to digest for a definite time and then titrated with 0.116 N barium hydroxide using 5 or 6 drops of a 1 per cent alcoholic solution of phenolphthalein as indicator. Toluene was used to prevent bacterial growth.

The radioactive solutions were prepared as follows: Radium emanation was collected in the usual way over mercury in a test tube, the amount present being estimated by  $\gamma$ -ray electroscope. This was transferred to a flask containing water, thoroughly shaken for a period of 20 minutes and the amount of emanation per cc. calculated. Varying amounts of this water were added to the lipase-ethyl butyrate mixtures in the amounts indicated.<sup>11</sup> The final volume of the mixture was made up to that of the control. The tubes or flasks used were at least half filled with liquid and tightly stoppered with well-fitting corks.

The results of the experiments can be found in the following tables. The activity of the enzyme is expressed in cc. of 0.116 N barium hydroxide necessary to neutralize the butyric acid formed. The values have been in all cases corrected for the acidity developed for the ethyl butyrate and lipase extract alone.

The data of the following tables show conclusively that no accelerating influence is exerted upon the lipase of the pig's liver or castor oil bean by radium emanation in the amounts used. On the contrary, inhibition of the enzymatic activity is suggested.

We acknowledge with pleasure our indebtedness to Dr. H. H. Young, through whose generosity the radium was placed at our disposal.

<sup>11</sup> Inasmuch as the flasks were not completely filled with liquid, a large proportion of the radium emanation would eventually be found in the air space above the solution.

TABLE 1.

Ten cc. saturated ethyl butyrate solution, 3 cc. undiluted liver extract, with either 2 cc. water or 2 cc. emanation water (room temperature).

MICROCURIES EMANATION	TIME IN MINUTES	cc. 0.116 N Ba(OH) <sub>2</sub> REQUIRED	
		Without R. E.	With R. E.
46	20	1.96	1.90
4	30	2.35	2.36
18	30		2.40
92	60	2.75	2.80
18	70	3.28	3.30
18	75	3.45	3.35
4	85	3.60	3.50

TABLE 2.

Ten cc. saturated ethyl butyrate solution, 2 cc. undiluted liver extract, with either 1 cc. water or 1 cc. emanation water (room temperature).

TIME IN MINUTES	cc. 0.116 N Ba(OH) <sub>2</sub> REQUIRED	
	Without R. E.	With R. E.
32	1.80	1.76
45	2.30	2.26
83	3.72	3.52
83	3.81	3.50

TABLE 3.

Ten cc. saturated ethyl butyrate solution, 2 cc. undiluted liver extract, 1 cc. water or 2 cc. emanation water.

TIME IN MINUTES	cc. 0.116 N Ba(OH) <sub>2</sub> REQUIRED	
	Without R. E.	With R. E.
25	0.86	0.82
102	1.98	1.65
161	2.20	1.70
161	2.30	2.50
230	2.80	2.07
230	3.00	
284	3.20	2.62

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TABLE 4.

Ten cc. saturated ethyl butyrate solution, 2 cc. undiluted liver extract, and 5 cc. water or 5 cc. emanation water allowed to digest 5 hours at room temperature.

cc. 0.116 N Ba(OH) <sub>2</sub> REQUIRED	
Without R. E.	With R. E.
3.98	4.22
4.31	4.21
4.16	3.42
4.05	4.13

TABLE 5.

0.5 cc. ethyl butyrate, 1 cc. liver extract (diluted with equal volume of water), and 25 cc. water or 25 cc. emanation water. Allowed to digest 20 hours at 38°.

MICROCURIES EMANATION	cc. 0.116 N Ba(OH) <sub>2</sub> REQUIRED	
	Without R. E.	With R. E.
17	5.40	4.80
17	6.00	4.85
85	5.70	4.70
85		4.30
0.3		5.95
0.3		5.75

TABLE 6.

0.5 cc. ethyl butyrate, 1 cc. liver extract (diluted with three volumes of water) and 25 cc. water or 25 cc. emanation water digested 44 hours at 38°.

MICROCURIES EMANATION	cc. 0.116 N Ba(OH) <sub>2</sub> REQUIRED	
	Without R. E.	With R. E.
3	6.70	5.00
3	6.75	5.10
3	6.60	5.10
3	6.60	5.00
0.17	6.75	5.90
0.17		5.80
0.02		6.50
0.02		6.70
0.002		6.70
0.002		6.65

TABLE 7.

0.5 cc. ethyl butyrate, 1 cc. liver extract (diluted three times), 25 cc. water or 25 cc. emanation water. Each flask contained 150 microcuries.

TIME IN HOURS	cc. 0.116 N Ba(OH) <sub>2</sub> REQUIRED	
	Without R. E.	With R. E.
22	4.65	4.30
22	4.80	4.20
48	6.25	5.70
48	6.10	5.80

TABLE 8.

0.5 cc. ethyl butyrate, 0.2 gm. castor bean, and 25 cc. water or 25 cc. emanation water. 25 cc. contained 150 microcuries.

TIME IN HOURS	cc. 0.116 N Ba(OH) <sub>2</sub> REQUIRED	
	Without R. E.	With R. E.
22	1.70	1.65
22	1.65	1.65
48	2.80	2.70
48	2.80	2.70

TABLE 9.

0.5 cc. ethyl butyrate, 0.5 gm. castor bean, and 25 cc. water or 25 cc. emanation water, digested 18 hours at 38°.

MICROCURI- ES EMANATION	cc. 0.116 N Ba(OH) <sub>2</sub> REQUIRED	
	Without R. E.	With R. E.
3	3.75	3.70
3	3.65	3.65
3	3.75	3.65
150		3.70
150		3.65

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TABLE 10.

0.5 cc. ethyl butyrate, 0.5 gm. castor bean, and 25 cc. water or 25 cc. emanation water, digested 50 hours at 38°. 25 cc. contained 80 microcuries.

cc. 0.116 N Ba(OH) <sub>2</sub> REQUIRED	
Without R. E.	With R. E.
6.35	6.15
6.40	6.15



## NOTE ON THE DETERMINATION OF AMINO-ACID NITROGEN IN URINE.

By S. R. BENEDICT AND J. R. MURLIN.

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(Received for publication, October 18, 1913.)

Something over a year ago the writers<sup>1</sup> reported a modified technique for the preparation of urines for the formalin titration method of Henriques and Sørensen. This consisted essentially in the removal of ammonia and other bodies by means of phosphotungstic acid, the clearing-out of the phosphotungstic acid by means of tribasic lead acetate and litharge and the final precipitation of the lead by a stream of hydrogen sulphide. The water-clear filtrate was then neutralized to litmus and titrated to the third stage end-point of phenolphthalein as directed by the original authors.

The results by this method in comparison with those of the "new" method of Henriques and Sørensen<sup>2</sup> were very much lower. With certain pure substances added to urines values very close to the theoretical were found.

Subsequently the list of pure substances was extended and it was found that a number of amino-acids were removed from the solution at times in whole or in part by the lead. This was notably true of aspartic acid and tyrosine, less so of glutamic acid and leucine. Much depended on the amount of basic lead acetate employed. After a time, however, it became apparent that it would be impracticable to control the quantity of lead and the conditions of temperature, etc., accurately enough to make the method of much value, and a different method of removing the phosphotungstic acid was sought. van Leersum<sup>3</sup> had employed KCl for this purpose in the amino-acid method which he devised

<sup>1</sup> Benedict and Murlin: *Proc. Soc. of Exp. Biol. and Med.*, ix, p. 109, 1912.

<sup>2</sup> *Zeitschr. f. physiol. Chem.*, lxiv, p. 120, 1909.

<sup>3</sup> van Leersum: *Biochem. Zeitschr.*, xi, p. 121.

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as a modification of the Pfaundler method. The first trials with the method as prescribed by van Leersum were not very successful. Traces of phosphotungstic acid (Merck) could always be found by the zinc test. Later it was found that the 5 per cent KCl solution would remove all of the acid provided a considerable excess of acid was left unprecipitated, and when this was not the case the addition of a little 10 per cent phosphotungstic acid in 2 per cent HCl would bring about complete precipitation. Under these circumstances also KCl could be used in substance and a water-clear filtrate could be readily obtained. Potassium salts however do not remove the phosphates and sulphates; consequently the attainment of an exact neutral point to litmus is very difficult. Going back to barium hydrate as a means of removing the phosphates and sulphates it was found that the barium would suffice also to precipitate the phosphotungstic acid. The whole procedure thus became very simple and as used in these laboratories now is as follows:

#### PROCEDURE.

1. Measure into a 500-cc. Erlenmeyer flask 200 cc. of a 24-hour human urine diluted to 2000 cc.
2. Add an equal quantity of 10 per cent phosphotungstic acid (Merck<sup>4</sup>) in 2 per cent HCl. Let stand at least three hours; better over night.
3. Pour off 250 cc. of the clear fluid; add 1 cc. of a 0.5 per cent solution of phenolphthalein, and barium hydrate in substance until the whole fluid turns decidedly pink. The barium hydrate should be added a very little at a time. Let stand one hour.
4. Filter off two 100-cc. samples (= 50 cc. urine).
5. Neutralize to litmus (Squibb's papers answer for all practical purposes) with  $\frac{N}{3}$  HCl.
6. Add 10-20 cc. neutral formalin and titrate cautiously to deep red color, *i.e.*, until the drop produces no additional color with  $\frac{N}{10}$  NaOH.
7. Correct by deducting the amount of  $\frac{N}{10}$  NaOH necessary to produce the same depth of color in an equal quantity of CO<sub>2</sub>-free water with the same quantity of neutral formalin added.

Some control tests are given below.

<sup>4</sup> Kahlbaum's preparation is a very different substance.

I. *Removal of ammonia by means of phosphotungstic acid.*

a. *Pure substances.* 1. 40 cc.  $\frac{N}{10}$  solution of aspartic acid + 20 cc.  $\frac{N}{10}$   $(\text{NH}_4)_2\text{SO}_4$  solution. 100 cc. 10 per cent phosphotungstic acid solution in 2 per cent HCl added in equal quantity. Stood over night. 5-10 grams KCl added to remove phosphotungstic acid. 100 cc. filtrate titrated 10.1; theory, 10.0.

2. 20 cc.  $\frac{N}{10}$  glycocholl + 20 cc.  $\frac{N}{10}$   $(\text{NH}_4)_2\text{SO}_4$ . 100 cc. Same procedure. 100 cc. filtrate titrated 9.5; theory, 10.0.

b. *Urines.* 1. 200 cc. urine containing 4.6 per cent  $\text{NH}_3\text{-N}$  + 200 cc. 10 per cent phosphotungstic acid in 4 per cent HCl stood over night. 20 cc. filtrate aerated by Folin method three hours into 10 cc.  $\frac{N}{10}$   $\text{H}_2\text{SO}_4$ . Titrated 10.0. Therefore all ammonia out.

2. 100 cc. urine containing 9.5 per cent  $\text{NH}_3\text{-N}$  + 100 cc. 10 per cent phosphotungstic acid in 4 per cent HCl. Stood over night. 20 cc. filtrate by Folin method three hours against 10 cc.  $\frac{N}{10}$   $\text{H}_2\text{SO}_4$  titrated 10.0.

3. 200 cc. urine containing 9.5 per cent  $\text{NH}_3\text{-N}$  + 50 cc. 10 per cent phosphotungstic acid in 4 per cent HCl. Stood over night. 20 cc. filtrate by Folin method four hours against 10 cc.  $\frac{N}{10}$   $\text{H}_2\text{SO}_4$  titrated 6.0. Therefore ammonia not all removed.

4. 100 cc. urine containing 9.4 per cent  $\text{NH}_3\text{-N}$  + 50 cc. 10 per cent phosphotungstic acid in 4 per cent HCl. Stood over night. 20 cc. filtrate by Folin method four hours titrated 5.0.

5. 100 cc. urine containing 13 per cent  $\text{NH}_3\text{-N}$  + 100 cc. 10 per cent phosphotungstic acid in 4 per cent HCl. Stood one and one-half hours. 20 cc. filtrate by Folin method eighteen hours titrated 9.9.

These results agree with those of Gumlich<sup>1</sup> in proving that at least an equal quantity of the 10 per cent phosphotungstic acid solution must be added to the urine and that the mixture should stand at least three hours.

II. *Removal of phosphotungstic acid by KCl.*

1. 100 cc. filtrate from urine (3) above + 100 cc. 5 per cent KCl. Stood two hours. Filtrate gives no blue color with zinc.

2. 100 cc. filtrate from same urine + 10 grams KCl in substance. Stood two hours. Filtrate clear of phosphotungstic acid.

3. a. 200 cc. urine + 200 cc. 10 per cent phosphotungstic acid in 2 per cent  $\text{H}_2\text{SO}_4$ .

b. 200 cc. same urine + 0.362 gram tyrosine + 200 cc. 10 per cent phosphotungstic acid.

c. 200 cc. same urine + 0.266 gram aspartic acid + 200 cc. 10 per cent phosphotungstic acid.

All stood for one week. Many crystals found on side of flask b and c. Warmed in water bath adding 200 cc. distilled water. Crystals dissolved. Stood four hours. Decanted:

400 cc. clear fluid from each flask + 10 grams KCl. Stood two hours.

<sup>1</sup> Gumlich: *Zeitschr. f. physiol. Chem.*, xvii, p. 13, 1893.

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100 cc. filtrate from *b* titrated 8.5 cc.; 100 cc. filtrate from *c* titrated 8.5 cc.

100 cc. filtrate from *a* titrated 5.3 cc., 5.3 cc.

Difference  $b-a = 3.2$  cc.:  $c-a = 3.2$  cc.

Theoretical difference, 3.33 cc.

### III. Removal of phosphotungstic acid by $\text{Ba}(\text{OH})_2$ .

1. *a.* 200 cc. urine (case of pernicious vomiting) + 200 cc. 10 per cent phosphotungstic acid in 2 per cent HCl.

*b.* Same containing 0.262 gram leucine. Stood 4 hours. 200 cc. filtrate + 50 cc. saturated solution  $\text{Ba}(\text{OH})_2$ . Stood one hour.

*b.* 100 cc. filtrate titrated 6.4 and 6.1 cc.  $\frac{N}{10}$  NaOH.

*a.* 100 cc. filtrate titrated 2.4 and 2.4 cc.  $\frac{N}{10}$  NaOH.

Difference, 4.0 and 3.7.

Theoretical difference, 4.0.

2. *a.* 200 cc. urine + 200 cc. phosphotungstic acid.

*b.* Same containing 0.326 gram tyrosine.

Stood three days. Crystals of tyrosine found on sides of flask. Added few drops concentrated HCl and warmed in water bath until crystals dissolved.

Phosphotungstic removed with  $\text{Ba}(\text{OH})_2$  in substance while keeping flask warm.

*b.* 100 cc. filtrate titrated 9.9 and 10.0.

*a.* 100 cc. filtrate titrated 4.8 and 4.9.

Difference, 5.1 and 5.1; theory, 5.0.

Aspartic acid added in similar quantity was partially removed by the phosphotungstic acid or possibly by the barium hydrate used in too great concentration.

Levene and Beatty<sup>6</sup> have shown that while amino-acids in general are not precipitated by phosphotungstic acid unless they are present in great concentration, there is considerable variation in this respect among the individual amino-acids.

Because of the great insolubility of tyrosine, leucine, aspartic acid, etc., in neutral medium, it is important not to let the filtrate stand after the neutral point is reached. The neutral formalin should be added at once.

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<sup>6</sup> Levene and Beatty: *Zeitschr. f. physiol. Chem.*, xlvii, p. 149, 1906.

## METABOLISM STUDIES ON COLD-BLOODED ANIMALS. II.

### THE BLOOD AND URINE OF FISH.

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(Received for publication, October 20, 1913.)

In a recent paper Folin and Denis<sup>1</sup> have published data regarding the non-protein nitrogen, urea and uric acid in the blood of a number of mammals and a few birds. More or less as a continuation of the work I have collected the blood of a number of the more common fish of the North Atlantic coast and in this blood have determined the total non-protein nitrogen, urea, ammonia, uric acid and creatine.

For the determination of the first four constituents the methods recently published by Folin and Denis<sup>2</sup> have been used; creatine was determined colorimetrically by the Folin method, the following procedure being employed.

Twenty grams of oxalated blood were poured slowly into 100 cc. of boiling  $\frac{N}{100}$  acetic acid solution and the mixture heated for two or three minutes until coagulation was complete; the solution was then filtered, the coagulum returned to the vessel in which the coagulation had been made and washed with about 200 cc. of boiling water. The original filtrate and the wash water were then combined, strongly acidified with acetic acid and rapidly evaporated down to a volume of about 5 cc. This residue was transferred to a small flask, the evaporating dish being first rinsed with 10 cc. of normal hydrochloric acid and then with a few cubic centimeters of water. The mouth of the flask was then loosely closed and the mixture heated for four hours on the boiling water bath. The color was developed in the usual way, but as a standard I used a solution of pure creatinine<sup>3</sup> in place of the customary half-normal potassium bichromate.

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<sup>1</sup> This *Journal*, xiv, p. 29, 1913.

<sup>2</sup> *Ibid.*, xi, p. 527, 1912; xiii, p. 469, 1913.

<sup>3</sup> *Ibid.*, xii, p. 149, 1912.

The blood was in every case taken from fish, just brought in from the traps and therefore still alive; in the case of the larger specimens the blood was removed from the heart by means of a needle and syringe, while in the case of the smaller animals it was collected from the caudal artery and vein. All figures presented are for whole blood in which coagulation had been prevented by the use of a little solid potassium oxalate. In the case of the larger fish (shark, goosfish, squeteague, etc.) not more than six animals were employed to secure the composite sample of blood, while in the case of the smaller ones (butterfish, mackerel, eel) from fifty to a hundred fish were used in order to obtain the requisite quantity of blood.

*Results of the examination of the non-protein nitrogen fraction of the blood of fish.*

(The figures represent milligrams per 100 grams of blood.)

	NON-PROTEIN NITROGEN	UREA NITROGEN	AMMONIA NITROGEN	URIC ACID	CREATINE + CREATININE
Dogfish ( <i>Mustelus canis</i> ).....	1000	800	1.4	0	4.0
Sand shark ( <i>Carcharias littoralis</i> ).....	1160	1000	2.5	0	4.0
Skate ( <i>Raia erniacea</i> ).....	1100	868	1.6	0	3.1
Alewife ( <i>Pinnolobus pseudoharengus</i> ).....	54	10	5.5	1.1	11.0
Butterfish ( <i>Poronotus triacanthus</i> ).....	50	9	5.1	1.4	16.0
Mackerel ( <i>Scomber scombrus</i> ).....	86	10	3.8	1.1	7.5
Squeteague ( <i>Cynoscion regalis</i> ).....	66	20	1.0	0.7	6.0
Menhaden ( <i>Brevoortia tyrannus</i> ).....	47	10	3.3	1.0	6.0
Summer flounder ( <i>Paralichthys dentatus</i> )..	46	8	1.1	0.8	5.0
Shad ( <i>Alosa sapidissima</i> ).....	90	16		1.1	
Bonito ( <i>Sarda sarda</i> ).....	90	17	3.8	1.0	
Goosfish ( <i>Lophius piscatorius</i> ).....	40	8	3.6	0.9	5.0
Eel ( <i>Anguilla crysypa</i> ).....	50	9	2.8	0.6	10.6

From the figures given in the above table it will be seen that by the new analytical method used I have in the case of the three elasmobranch fishes examined (shark, dogfish and skate) given quantitative confirmation of the earlier observations<sup>4</sup> regarding the large amount of urea contained in the blood of these animals. In the case of the teleosts, however, the percentage of

<sup>4</sup> Schröder: *Zeitschr. f. physiol. Chem.*, xiv, p. 576, 1890; Baglioni: *Centralbl. f. Physiol.*, xix, 1905.

non-protein nitrogen accounted for by the urea fraction is much smaller than in the blood of man or of any mammal so far examined. In the series of determinations of urea in the blood of seven different kinds of animals (rabbit, sheep, pig, horse, monkey and beef) recently made with the same method by Folin and Denis;<sup>5</sup> and in the large number of urea determinations on normal human blood made by the same investigators it was found that the urea nitrogen fraction accounted for about 50 to 60 per cent of the non-protein nitrogen of the blood. In the case of bird blood (chicken, duck and goose) it appears, however, that the urea-nitrogen fraction accounted for only 25 to 30 per cent of the non-protein nitrogen.

The low urea content of bird blood agrees well with what is known regarding the small percentage of the total urinary nitrogen of these animals which is accounted for by urea. Regarding the urinary urea of fish but little is known. In a recent paper<sup>6</sup> I have reported analyses of the urine of the dogfish in which urea nitrogen amounted to from 80 to 89 per cent of the total nitrogen. The blood of the elasmobranchs differs, however, so markedly from that of the teleosts that it is to be expected that the urine of the two classes would also show marked dissimilarity.

Below is given the result of an examination of a composite sample of the urine of the goosfish (*Lophius piscatorius*). This urine was secured from the bladders of six fish about one hour after death, and was examined on the day of collection. In general it may be said that this urine was a clear, pale yellow fluid, with an acid reaction and a markedly fishy smell. On heating to boiling a heavy coagulum of earthy phosphates was produced which dissolved on the addition of dilute acetic acid.

*Analysis of a composite sample of urine from six goosfish.*

Specific gravity 1.016. Albumen and reducing sugar absent.

	Mgm. per liter	Per cent of total N		Mgm. per liter
Total N.....	830		Phosphates (as $P_2O_5$ )...	440
Urea N.....	120	14.4	Chlorides (as NaCl).....	10800
Ammonia N.....	12	2.7	Total sulphur.....	108
Uric acid N.....	1	0.1	Inorganic sulphates (as S)	92
Creatinine N.....	7	0.8		
Creatine N.....	140	16.6		

<sup>5</sup> This *Journal*, xiv, p. 29, 1913.

<sup>6</sup> *Ibid.*, xiii, p. 225, 1912.

From this examination of the urine of the goosfish, a representative teleost, it is apparent that the small percentage of urea in the blood is also coincident with a small urea excretion in the urine.

The large percentage of undetermined nitrogen in this urine is also noteworthy. It occurred to me that this might be due to the presence in large amounts of amines. Qualitative tests, however, have not confirmed this hypothesis. Another interesting point brought out is the fact that apparently in the urine of a bony fish creatinine is almost entirely replaced by creatine. A somewhat similar condition has been shown to exist in the bird.<sup>7</sup> It should be remembered, however, that the goosfish is a voracious eater, as much as five pounds of food being frequently found in its stomach and as this food consists of small fish the goosfish must undoubtedly consume a considerable quantity of creatine, a fact which may account in part at least for the large amount of creatine contained in the urine. The high dilution of this urine is also not without interest as it would seem to support the theory that in the fish nitrogenous waste products may be eliminated in part by some organ other than the kidney.

The figures obtained for the ammonia nitrogen fraction of the blood are surprisingly large. A number of ammonia determinations made by the same method by Folin and Denis<sup>8</sup> showed that the quantity of ammonia in the systemic blood of cats amounts to not more than 0.1 or 0.2 mgm. per 100 grams of blood. Further observations (unpublished) on the ammonia content of normal and pathological human blood have shown that here too ammonia is present to the extent of only a fraction of a milligram per 100 grams of blood.

In connection with the high ammonia content of fish blood it is not inappropriate to mention the well-known experiments of Cohnheim on the deaminizing power of the intestinal mucosa,<sup>9</sup> in which work, although able to demonstrate the deaminizing power of the surviving intestines of fish, he met with small success when the intestines of cats and dogs were employed.

<sup>7</sup> Paton: *Journ. of Physiol.*, xxxix, p. 485, 1910.

<sup>8</sup> This *Journal*, xi, p. 161, 1912.

<sup>9</sup> *Zeitschr. f. physiol. Chem.*, lix, p. 239, 1909; also lxi, p. 189, 1909.



My results on the uric acid content of the blood and urine of fish are somewhat difficult to explain. As will be noted I have been unable to find more than a minute trace of uric acid in the blood of any of the elasmobranchs examined, while in the blood of all the teleosts it exists in moderate amounts. Uric acid was found in small amounts in the urine of both classes of fish. These findings are contrary to those of Baglioni<sup>10</sup> who states that in the blood of the dogfish there is to be found a larger quantity of uric acid than in the urine.

<sup>10</sup> *Centralbl. f. Physiol.*, xx, p. 105, 1906.



## NOTE ON THE TOLERANCE SHOWN BY ELASMOBRANCH FISH TOWARDS CERTAIN NEPHROTOXIC AGENTS.

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Since the initial publications by Schlayer and his associates in 1907 a large number of investigations have been carried out dealing with different phases of the experimental nephritis produced by the injection of various organic and inorganic nephrotoxic agents. In these experiments rabbits, dogs and cats have been the animals invariably used.

In several recent publications<sup>1</sup> it has been shown that in the case of cats and rabbits in whom nephritis had been experimentally produced the non-protein nitrogen of the blood may be increased to many times the normal amount. In these experiments our experience has been that in cases in which the non-protein nitrogen content of the blood was greatly elevated the prognosis was bad.

The elasmobranch fish occupy a unique position with regard to the non-protein nitrogen content of the blood. In these animals this fraction amounts to about 1000 mgm. per 100 grams blood, of which about 80 per cent is urea nitrogen; the tissues likewise contain large amounts of urea, which substance has also been shown to be present in considerable quantities in the intestinal contents, and in the bile.<sup>2</sup> Elimination by way of the kidneys is small, only 20 to 50 mgm. of urea nitrogen per kilo being excreted in the urine by the starving dogfish in twenty-four hours.<sup>3</sup> In view of the above facts it occurred to me that it might be interesting to see whether the elasmobranch fishes might not be

<sup>1</sup> Folin, Karsner and Denis: *Journ. of Exp. Med.*, xvi, p. 789, 1912; Frothingham, Fitz, Folin and Denis: *Arch. of Int. Med.*, xii, p. 245, 1913.

<sup>2</sup> Van Slyke and White: this *Journal*, ix, p. 209, 1911.

<sup>3</sup> Denis: *ibid.*, xiii, p. 225, 1912.

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able to withstand the administration of relatively enormous doses of renal poisons.

For all experiments the smooth dogfish (*Mustelis canis*) was used. During the experiment the animals were kept in large tanks supplied with running sea water and were fed every second day with fish; in many cases, however, food was refused.

As the number of tanks available was somewhat limited I have confined myself to the study of two well-known nephrotoxic agents, *i.e.*, uranium nitrate and potassium chromate. These substances were administered by means of subcutaneous injections into the flank, each dose being injected into several different places.

As will be seen by inspection of Table I, uranium nitrate may be administered to the dogfish in doses as large as 80 mgm.<sup>4</sup>

TABLE I.  
*Uranium nitrate experiments.*

NUMBER OF FISH	WEIGHT IN GRAMS	URANIUM NITRATE GIVEN, AS MILLIGRAMS PER KILO	REMARKS
1	1010	64	Killed 5 days after injection.
2	1015	64	Killed 5 days after injection.
3	2050	32	Killed 5 days after injection.
4	1012	64	Killed 5 days after injection.
5	1500	53	Killed 5 days after injection.
6	1500	53	Killed 5 days after injection.
7	1020	80	Killed 5 days after injection.
8	800	100	Killed 5 days after injection.
24	1350	60	Killed 5 days after injection.
31	700	80	Killed 6 days after injection.
32	1550	51	Killed 7 days after injection.
62	1000	80	Killed 6 days after injection.
63	1200	66	Killed 6 days after injection.
64	1350	60	Killed 8 days after injection.
65	1550	50	Killed 8 days after injection.
23	750	106	Died 68 hours after injection.
27	630	126	Died 26 hours after injection.
29	570	140	Died 43 hours after injection.
28	510	156	Died 37 hours after injection.

<sup>4</sup> Rabbits are frequently rendered anuric by doses of 1 to 3 mgm. of uranium nitrate per kilo of body weight; dogs and cats are somewhat less sensitive, but even with these animals serious symptoms are obtained by the administration of 5 to 10 mgm. per kilo.

per kilo of body weight without ill effects. All animals killed remained in excellent condition during the entire experimental period.

A similar series of experiments was undertaken in which potassium chromate was administered. The results are given in the following table, and show that here again the dogfish appears to be very resistant towards this class of poisons.

TABLE II.  
*Potassium chromate experiments.*

NUMBER OF FISH	WEIGHT IN GRAMS	POTASSIUM CHROMATE GIVEN AS MILLIGRAMS PER KILO	REMARKS
37	1350	48	Killed 5 days after injection.
38	1150	55	Killed 6 days after injection.
39	1080	59	Killed 6 days after injection.
40	1090	59	Killed 6 days after injection.
19	1020	94	Killed 5 days after injection.
20	1080	88	Killed 5 days after injection.
18	600	166	Died 40 hours after injection.

An attempt was also made in a few cases to determine whether the accumulation of nitrogenous waste products could be demonstrated in dogfish in whom nephritis had been experimentally induced. In order to obtain an idea of the average amounts of total non-protein nitrogen and urea nitrogen present in the blood of the dogfish, samples of blood were secured from twenty different animals, care being taken to choose fish of varying sex, weight and age. In these samples urea and total non-protein nitrogen were determined by the methods of Folin and Denis.<sup>5</sup> The maximum, minimum and average values found were as follows (results are expressed as milligrams per 100 grams of blood):

	Maximum	Minimum	Average
Non-protein nitrogen.....	1240	900	1000
Urea nitrogen.....	960	713	800

As will be seen by the results presented in Table III no accumulation could be demonstrated in dogfish to whom large doses of uranium nitrate and potassium chromate had been given, a

<sup>5</sup> This *Journal*, xi, p. 527, 1912.

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TABLE III.

*Non-protein nitrogen and urea in the blood of nephritic dogfish.*  
(Milligrams per 100 grams blood.)

LABORATORY NUMBER OF FISH	UREA NITROGEN	TOTAL NON-PROTEIN NITROGEN	LABORATORY NUMBER OF FISH	UREA NITROGEN	TOTAL NON-PROTEIN NITROGEN
2	757	1000	32	765	990
3	800	1050	28	713	900
4	840	1120	19	687	875
8	880	1250	39	713	925
31	800	1000	40	687	920

result not surprising if we take into consideration the small elimination by the kidneys, and the apparent ability of these animals to utilize the liver and perhaps the intestine as an excretory organ.

An attempt was made with a number of animals to collect samples of urine by means of a cannula tied in the urinary papilla; in every case, however, anuria had apparently set in by the third or fourth day so that I am unable to report the results of any urine examinations.

## UREA FORMATION IN THE LIVER.<sup>1</sup>

### A STUDY OF THE UREA-FORMING FUNCTION BY PERFUSION WITH FLUIDS CONTAINING (a) AMMONIUM CARBONATE AND (b) GLYCOCOLL.

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(Received for publication, October 21, 1913.)

#### INTRODUCTION.

It is a desirable thing to be able to localize special functions to definite sites. Attempts of this sort are frequently made, and in a large number of cases it has been the liver to which attention has been directed. There seem to be a variety of reasons for this. One is that the location of this organ is such as to suggest that it serves the function of protecting the organism in general from toxic substances entering the circulation from the digestive tract; another, that a considerable amount of evidence of such a protective function exists; still another, that its size is taken, and probably rightly, to be more or less of an index of its importance. Undoubtedly it has often served as the scapegoat where ignorance has existed, especially from a clinical standpoint. Perhaps, also, the fact that the liver is a comparatively easy organ to perfuse by itself has something to do with its popularity. That its importance as a specific site of metabolic processes has been overestimated is shown by the decrease in the rôle ascribed to it in carbohydrate metabolism in past years. Fischler and Bardach<sup>2</sup> have apparently demonstrated that normal utilization of sugar can take place when it is severely injured.

Among the processes at various times attributed to the liver, few have been so prominent and so much discussed as that of the formation of urea, especially from ammonium salts and from amino-acids.

<sup>1</sup> Aided by a grant from the Rockefeller Institute for Medical Research.

<sup>2</sup> *Zeitschr. f. physiol. Chem.*, lxxviii, p. 435, 1912.

Concerning the ability of the liver to synthesize urea from ammonium salts of organic acids which are oxidized in the body to carbon dioxide and water, there can be no doubt. The first demonstration of this by v. Schroeder,<sup>3</sup> including the isolation and identification of urea nitrate, took place and was confirmed long ago. The purpose of such a function appears definite in the light of recent investigations by Folin and Denis,<sup>4</sup> which demonstrate that the ammonia of the portal blood originates largely in the large intestine and is therefore chiefly a bacterial product. It is easy to understand why it should be rendered innocuous before being distributed to the tissues. That the liver is the sole site of this process is far from being a proved fact. The old assumption that the increase in the excretion of ammonia in disturbances of the liver is due directly to hepatic insufficiency need not be considered, since the evidence against it is too great (Muenzer<sup>5</sup> and others).

Quite otherwise, however, stand the facts concerning urea formation from amino-acids. According to the view which appeared most probable until recently, the amino-acids are deaminized somewhere between the lumen of the intestine and the liver, the nitrogen being carried in the form of ammonia to the liver, there to be converted into urea, or else being immediately resynthesized into protein in the intestinal wall. Various discoveries by Folin and Denis<sup>6</sup> have greatly modified the status of this question: (1) the demonstration of the rapid distribution of amino-acids *as such* to the tissues, *without* immediate alteration, the storage of the same in a "nitrogen reservoir" (at least partly in the muscles) and their later conversion into urea, and (2) the finding of ammonia in the portal (and systemic) blood in concentrations much below those previously supposed to exist, with the major part of it coming from the large intestine. These findings make it quite unnecessary to assume that either deaminization or resynthesis occurs in the intestinal wall.

The only direct evidence now existing of the occurrence of urea

<sup>3</sup> *Arch. f. exp. Path. u. Pharm.*, xv, p. 364, 1882.

<sup>4</sup> *This Journal*, xi, p. 161, 1912.

<sup>5</sup> *Deutsch. Arch. f. klin. Med.*, lii, p. 199, 1894; *Arch. f. exp. Path. u. Pharm.*, xxxiii, p. 164, 1894.

<sup>6</sup> *This Journal*, xi, pp. 87, 161, 1912; xii, p. 141, 1912.



formation directly from amino-acids in the liver is that of Salaskin,<sup>7</sup> which has stood for fifteen years with no great amount of criticism except to the effect that he did not isolate urea. His results, obtained by the perfusion of the liver with amino-acids, which showed an increase in urea by the method of Schöndorff, can hardly be accepted. In the first place he gives only the percentage of urea (amid-nitrogen) in the perfusing fluid, without stating the amount of fluid recovered. It is therefore impossible to know how much urea (total amount) there really was in the fluid at the end of any experiment, although with the amounts used (about 1000 cc.) it is not likely that any large proportion of the volume disappeared by concentration. The point in his results that stands out most prominently, however, is the fact that in all but one of four experiments in which he made analyses in the middle of the perfusion (including both of the two with glycocoll) he obtained a *greater increase in urea in the second than in the first half of the experiment* (lasting about three or four hours). It is quite inconceivable that such a result could be obtained with an organ removed from the body in an experiment begun fifteen to thirty-five minutes after the death of the animal and lasting more than three hours. If he did actually find such an increase in urea as his results indicate, a certain amount of it at least can be explained only as a result of abnormal post-mortem change. The liver certainly could not be more active in the second half of the experiment than in the first. The possibility suggests itself that ammonia was formed from amino-acids by autolysis or bacteria, or both. Any decisive information for or against such a possibility need hardly be attempted now, inasmuch as no one knows the nature, the abundance or the activity of the flora inhabiting his material, or the relationships of factors governing autolysis therein. If the above suggestion be the correct one, the increase in urea is easily explained. Certain it is that a liver, after a three-hour perfusion, is far from normal in appearance (edema, hemorrhages, excessive fragility, etc.), so that degenerative changes are quite conceivable. That ammonia is formed in the course of a comparatively short perfusion is evident from our results below. The assumption of such a formation of ammo-

<sup>7</sup> *Zeitschr. f. physiol. Chem.*, xxv, p. 128, 1898.

nia in the experiments of Salaskin might possibly explain his failure to get any evidence of urea formation in the perfusion of muscle, inasmuch as urea formation from ammonia has never been demonstrated there, although that it may occur is still quite possible. Since Salaskin's figures cannot be interpreted as meaning that all the increase in the urea values found represents urea (amid-nitrogen) formation from amino-acids, it is impossible to prove that any of it does.

Another possible explanation of the discrepancy between the results of Salaskin and the quite different ones obtained by us (see below) lies in a consideration of the methods employed. It is interesting to note that analyses of samples of the same material (intestinal mucosa) by Salaskin and Kowalewsky,<sup>8</sup> using the Schöndorff and the Mörner-Folin methods (applied to tissue analysis) showed 32 mgms. per 100 grams by the former, and only 14 mgms. by the latter, whereas on the other hand, urea determinations by the Schöndorff method for urine tend to give lower results (Folin<sup>9</sup>). Furthermore, the normal urea-content of human blood (expressed as nitrogen) by the Schöndorff method has been found to be 23 to 28 mgms. per 100 cc. by v. Jaksch<sup>10</sup> (Schöndorff<sup>11</sup> earlier, in one case, found 28.5 mgms.), while by the method of Folin and Denis<sup>12</sup> it is uniformly 11 to 13 mgms. per 100 cc. The figures of v. Jaksch and of Schöndorff for the urea nitrogen are practically the same as those obtained by Folin and Denis for the total non-protein nitrogen (22 to 26 mgms. per 100 cc.). Therefore, it is extremely probable that the Schöndorff figures include something not hydrolyzed by the method of Folin and Denis. It would appear that this substance, whatever it may be, is present in relatively greater concentration in the blood than in the urine.

Leaving out of consideration experiments with organ extracts and the like *in vitro* (Jacoby, Gottlieb, Lang and others), which, as suggested by Folin and Denis,<sup>13</sup> are quite unacceptable (since

<sup>8</sup> *Zeitschr. f. physiol. Chem.*, xlii, p. 410, 1904.

<sup>9</sup> *Ibid.*, xxxii, p. 504, 1901.

<sup>10</sup> *Internat. Beitr. z. inn. Med.*, i, p. 197, 1902. Quoted by Maly, xxxii, p. 265, 1902.

<sup>11</sup> *Pflüger's Archiv*, lxxiv, p. 307, 1899.

<sup>12</sup> *This Journal*, xiv, p. 29, 1913.

<sup>13</sup> *Ibid.*, xi, p. 527, 1912.

the abnormal production of ammonia first is not excluded), the only other evidence of urea formation from amino-acids in the liver has been derived from the results of operations upon the liver (Eck fistula, extirpation, etc.) and from liver disease (in human beings, and experimentally in animals). In the former case the evidence is based on urine analyses made either shortly after the operation, or later during a period of acute intoxication as a result of feeding meat, etc.; in either event the animal is distinctly abnormal (not solely with respect to its liver) and we believe that conclusions drawn from such results have no importance whatsoever as bearing upon this question, for it is certainly impossible to confine a general acute intoxication to a single organ. For instance, it has frequently been entirely disregarded that many of the procedures used in such experiments produce a disturbance of renal activity, and no one knows how great a part the nephritic element has played in such results, for it is now well known that the absence of albumen and casts is far from being an absolute proof of normal renal function.

As far as urine analyses in liver disease are concerned, Fawitzky<sup>14</sup> demonstrated nearly a quarter of a century ago that the chief cause of the earlier obtained low urea values was purely a result of the low protein intake. When the true significance of the increased excretion of ammonia in such conditions was brought to light (Muenzer<sup>15</sup>), the interest attached to this product was transferred to the amino-acids.

The evidence from the examination of the urine in cases of liver disease, in recent times, has consisted chiefly in demonstrations, by methods of very varying degrees of accuracy, of the existence of an increased excretion of amino-acid nitrogen, and of the recovery of amino-acids from the urine after they have been fed in amounts supposed to be largely destroyed by the normal individual (the latter primarily by Glaessner<sup>16</sup>). In the first place it is quite impossible to say, in any case of liver disease, that the liver is the sole site of functional disturbance, and therefore, no matter how important such findings may prove to be for the clinical diagnosis of such affections, it is not permissible to draw definite conclusions

<sup>14</sup> *Deutsch. Arch. f. klin. Med.*, xlv, p. 429, 1889.

<sup>15</sup> *Loc. cit.*

<sup>16</sup> *Zeitschr. f. exp. Path. u. Ther.*, iv, p. 336, 1907.

as to the normal functions of the liver from urinary findings in disease-complexes in which the liver merely dominates the clinical and the anatomical pictures. The assumption that the increased excretion of amino-acids in cirrhosis, for example, is due in part at least to autolysis is supported by Samuely's<sup>17</sup> finding of a similar phenomenon in lobar pneumonia at the time of the crisis (isolation of abnormally large amounts of  $\beta$ -naphthalinsulphoglycine), although before this result appeared it was stated that the absence of such an increase in pneumonia was against the view that autolysis was concerned. As a matter of fact, the results of investigations of this nature in liver disease so far have been exceedingly variable. For example, Bergell and Blumenthal<sup>18</sup> found a normal nitrogen partition and normal behavior of 20 grams of alanine in acute yellow atrophy. Masuda<sup>19</sup> also has obtained results quite different from those of Glaessner. Certainly there is no functional liver test that gives results sufficiently constant for definite conclusions to be based on them, especially since the conditions in which they are the most constant are acute toxic states (when organs other than the liver are similarly damaged) and advanced stages of more or less localized, extensively destructive diseases of the liver (in which the patient is far from being functionally normal otherwise than as to his liver). Even in the most extensive of these, viz., acute yellow atrophy and phosphorus poisoning, a number of cases have been reported in which normal amounts of urea and other nitrogenous constituents (allowing when necessary for "neutralization ammonia") were excreted, and in some of these instances amino-acids administered were destroyed to a normal extent (Rosenheim,<sup>20</sup> Muenzer,<sup>21</sup> Badt,<sup>22</sup> Richter,<sup>23</sup> Neuberg and Richter,<sup>24</sup> Bergell and Blumenthal,<sup>25</sup> Ishihara<sup>26</sup> and others). It is evident, therefore, from the numerous inconsisten-

<sup>17</sup> *Zeitschr. f. physiol. Chem.*, xlvii, p. 377, 1906.

<sup>18</sup> *Charité-Annalen*, xxx, p. 19, 1906.

<sup>19</sup> *Zeitschr. f. exp. Path. u. Ther.*, viii, p. 629, 1911.

<sup>20</sup> *Zeitschr. f. klin. Med.*, xv, p. 441, 1888.

<sup>21</sup> *Loc. cit.*

<sup>22</sup> *Centralbl. f. klin. Med.*, xiii, p. 251, 1891.

<sup>23</sup> *Berl. klin. Wochenschr.*, 1896, p. 454.

<sup>24</sup> *Deutsch. med. Wochenschr.*, 1904, p. 499.

<sup>25</sup> *Loc. cit.*

<sup>26</sup> *Biochem. Zeitschr.*, xli, p. 315, 1912.

cies in the literature, that the time is not yet when the nature of normal processes occurring in the liver can be definitely settled by the examination of the urine in cases of liver disease. Such investigations will be of more value when they can be carried on from another view-point, for when the physiology of the liver as learned by more direct methods shall become better understood, their results may be of importance in determining more nearly what actually occurs in such disturbances.

On the other side, an important piece of evidence in favor of the assumption that the liver is not a special site of urea formation from amino-acids has appeared in the work of Folin and Denis,<sup>27</sup> in which they have failed to find, by a method which as they say could hardly fail to show it if it existed, any difference in the urea-content of the hepatic venous blood and of blood from other parts of the body while non-protein nitrogen from amino-acids or Witte's peptone was being absorbed from the small intestine and the urea content of the blood increasing.

#### EXPERIMENTAL.

Our experiments consist in the perfusion of the livers of rabbits and cats with defibrinated blood, with or without the addition of serum and Ringer's solution or of the latter alone. The methods used have permitted the employment of comparatively small quantities of fluid (usually about 100 cc.). In all the cat experiments the blood has been that of the animal whose liver was used; in all, the fluid has been from the same species. We have performed experiments of two kinds; with the addition of (1) commercial ammonium carbonate, and (2) glycocoll, to the perfusing fluid.

#### *Method.*

The analyses were made in duplicate. Total non-protein nitrogen, urea and ammonia were all determined by the recently devised methods of Folin and Denis.<sup>28</sup> The figures given as urea nitrogen represent, of course, everything hydrolyzed in a constant boiling mixture consisting chiefly of potassium acetate at about

<sup>27</sup> This *Journal*, xii, p. 141, 1912.

<sup>28</sup> *Ibid.*, xi, p. 527, 1912.

150°C. in ten minutes, minus the ammonia nitrogen separately determined.

Technically, the experiments were arranged so that small quantities of perfusing fluid could be used, passing continuously and repeatedly in the same direction at an approximately constant temperature. The fluid was accurately measured at the beginning and end of the experiment and was usually about 100 cc. The temperature was usually at 38°C., but occasionally momentarily rose to as much as 40° and very rarely sank to 35°. The accompanying diagram shows the scheme adopted for the use of small quantities of fluid, the bottles being of 125 cc. capacity with wide necks. Perforated rubber stoppers were used and rubber tubing of 2 mm. caliber. The fluid passed out of one bottle through the liver and into the other bottle, then by throwing over the rocker valve, the filled bottle was made the supply bottle, the fluid continuing to flow in the same direction without noticeable interruption. The tube conducting fluid away from the bottles was connected with a coil of glass tubing in a leaden box containing water heated by an electric stove. From the coil the fluid passed to a T-tube in which the bulb of a thermometer was placed so that one arm connected with the heating coil, one arm with the thermometer, and one arm with a rubber tube 10 cm. in length which led to a cannula in the portal vein. The tubing from the T-tube to the portal vein, as well as the liver, trunk of the animal and several centimeters of tubing leading away from the inferior vena cava were contained in a double walled tin box, in which a temperature of about 40°C. was maintained by means of an electric light bulb.

All animals were bled to death before using the liver for perfusion. In the case of the cat, the animal was etherized and bled from a cannula placed in the carotid artery. In this way sufficient blood could be collected from one animal to serve for the experiment with its own liver. The blood was rapidly defibrinated in a flask with glass beads and the blood mixed with the other materials as described above. As soon as bled, the abdomen and thorax were opened, glass cannulae placed in the portal vein and in the inferior vena cava immediately above the diaphragm. The aorta and vena cava were ligated beneath the diaphragm and the

lower part of the trunk and upper part of the thorax severed. The remaining part of the trunk including liver and intestines was wrapped in towels soaked in hot salt solution (0.85 per cent NaCl), placed in the tin box and connected with the tubing so that the perfusing fluids entered the portal vein. This procedure occupied from ten to twenty minutes.

In the case of the rabbit, the same technique was used except that usually the blood of two animals had to be mixed in order to obtain the proper amounts. These animals were bled by a rapid severing of the femoral artery so that a minimum of ether was used. The animals were dead before being opened for exposure of the liver.

The length of time occupied in the perfusion was in most instances about one hour. The definite results obtained with ammonium carbonate in this length of time, combined with the distinctly abnormal appearance of the liver after several hours' perfusion and the incongruous results obtained by Salaskin appear to justify the employment of short experiments. In every case the blood was removed from the liver by previously perfusing with Ringer's solution, therefore it was not considered necessary to run the fluid through before making the first analysis. The substance used in each case was added in aqueous solution to the fluid and the two thoroughly mixed by shaking. Samples were then taken for analysis (5 cc. for precipitation with acetone-free methyl alcohol, 4 to 10 cc. for duplicate ammonia analyses) and the fluid poured into one of the bottles. Samples were again taken at the end of the experiment (and in one case during its course). The total quantities of the various substances analyzed were calculated from the amounts of fluid put in at the beginning and removed at the end of each experiment in those cases in which obvious loss, as a result of leakage, etc., did not occur. The remarkable constancy of the total non-protein nitrogen in most of the experiments in which there was no such loss (Experiments 10 and 11 are exceptions) indicates how slight must have been any washing out from or absorption by the liver during their course. Naturally the fluid left in the liver could not be added to that recovered, nor was its amount determined, but there can be no doubt that calculations based upon the total amount recov-

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ered are of more value than mere percentages. Slowtzoff and Ssobolew<sup>29</sup> furthermore found only 2.0 to 5.6 per cent of blood in the livers of human cadavers by colorimetric determination.

### I. Ammonium carbonate.

EXPERIMENT 1. Normal rabbit. Fluid: 23 cc. rabbit serum, 36 cc. defibrinated rabbit blood, 31 cc. Ringer's solution and 101 mgms. of commercial ammonium carbonate. Sixty-five minutes, nineteen times. In, 100 cc. Out, 115 cc.

	MILLIGRAMS PER 100 CC.		TOTAL AMOUNT IN MILLIGRAMS	
	Total non-protein nitrogen	Ammonia nitrogen	Total non-protein nitrogen	Ammonia nitrogen
Before.....	62	22	62	22
After.....	54	7	62	8

EXPERIMENT 2. Normal rabbit. Fluid: 23 cc. rabbit serum, 20 cc. defibrinated rabbit blood, 57 cc. Ringer's solution and 103 mgms. ammonium carbonate. One hour, forty times. In, 100 cc. Out, 98 cc. (diluted to 155 cc.).

	MILLIGRAMS PER 100 CC.		TOTAL AMOUNT IN MILLIGRAMS	
	Total non-protein nitrogen	Ammonia nitrogen	Total non-protein nitrogen	Ammonia nitrogen
Before.....	66	24	66	24
After.....	43	5	67	8

In the following three experiments (3, 4 and 5) accidents caused the loss of considerable amounts of fluid, therefore the total recovery is unknown.

EXPERIMENT 3. Normal rabbit. Fluid: 18 cc. rabbit serum, 53 cc. defibrinated rabbit blood, 9 cc. Ringer's solution, 20 cc. normal saline solution, 71 mgms. glycocoll and 45 mgms. ammonium carbonate. In, 100 cc.

	MILLIGRAMS PER 100 CC.	
	Total non-protein nitrogen	Ammonia nitrogen
Before.....	62	14
After.....	60	4

<sup>29</sup> *Biochem. Zeitschr.*, xxxi, p. 234, 1911.



EXPERIMENT 4. Liver of rabbit in which biliary cirrhosis had been produced by ligation of the common bile-duct five weeks before (Richardson<sup>30</sup>). Fluid: 63 cc. defibrinated rabbit blood, 37 cc. Ringer's solution and 104 mgms. ammonium carbonate. Thirty-two minutes, five times. In, 100 cc.

	MILLIGRAMS PER 100 cc.			
	Total non-protein nitrogen	Urea nitrogen	Ammonia nitrogen	* Rest nitrogen
Blood.....	43	18	Trace <sup>31</sup>	25
Fluid before.....	51	11 <sup>32</sup>	24	16
Fluid after.....	62	26	7	29

The ammonia fell from 47 to 11 per cent of the total non-protein nitrogen (decrease of 77 per cent.) \* The urea increased from 22 to 42 per cent of the total non-protein nitrogen (an increase corresponding to 56 per cent of ammonia lost). Although a perfectly definite increase in urea (amid nitrogen) is seen, 44 per cent of the lost ammonia was not recovered as urea. The two possibilities are: (1) that the remainder of the ammonia was retained in the liver, and (2) that it was converted into something other than urea. The results of Experiment 6 throw some light upon this.

EXPERIMENT 5. Normal rabbit. Starved forty-eight hours. Fluid: 65 cc. defibrinated rabbit blood, 35 cc. Ringer's solution and 105 mgms. ammonium carbonate. Twenty-eight minutes, eleven times. In, 100 cc.

	MILLIGRAMS PER 100 cc.	
	Total non-protein nitrogen	Ammonia nitrogen
Before.....	54	21
After.....	60	7

<sup>30</sup> *Journ. of Exp. Med.*, xiv, p. 401, 1911.

<sup>31</sup> Quantitative ammonia determinations were not made when 10 cc. of the Nesslerized solution could not be read in the ordinary colorimeter. Where a "trace" is reported, however, it is certain that there was considerably less than 0.5 mgm. in 100 cc. of the blood. This and other analyses of normal blood from both rabbits and cats confirm the findings of Folin and Denis, viz., that the ammonia in the systemic blood of normal animals is present only in traces.

<sup>32</sup> Calculated from the amount found in the defibrinated blood before anything was added to it, on the basis of the dilution.

EXPERIMENT 6. Normal cat. Starved twenty-four hours. Fluid: 40 cc. defibrinated cat blood, 35 cc. Ringer's solution and 107 mgms. ammonium carbonate dissolved in 4 cc. water. One hour, forty-five times. In, 80 cc. Out, 92 cc.

	MILLIGRAMS PER 100 CC.				TOTAL AMOUNT IN MILLIGRAMS			
	Total n.p. nitrogen	Urea N	Ammonia N	Rest N	Total n.p. nitrogen	Urea N <sup>23</sup>	Ammonia N	Rest N
Blood.....	51	23	Trace	31	20	9	Trace	11
Fluid before.....	52		28		42		23	
Fluid after	48	15	7	26	44	14	6	24

The last experiment, in which a complete recovery was made, shows the same thing as Experiment 4. Seventy-two per cent of the ammonia disappeared, but only 26 per cent of the lost ammonia was recovered as urea. The constancy of the total non-protein nitrogen here, as in other experiments in which practically everything was recovered, is greatly against the assumption that any appreciable amount of nitrogen, in the form of ammonia or otherwise, has been retained in the liver. The conclusion of Kowalewsky and Markewicz<sup>24</sup> from their perfusion experiments, that the ammonia is deposited in the perfused organ, is based upon two experiments with muscle, in which increases of 4.8 and 7.8 mgms. of ammonia per 100 grams, respectively, were found, after perfusion with blood containing about 14 mgms. per 100 grams; the method (distillation with magnesia) is certainly not accurate for blood, and is therefore even less likely to be so for tissues. If the in-

<sup>23</sup> The determination of urea in the presence of comparatively large amounts of ammonia by the method used has not given satisfactory results, the loss occurring, as far as we now know, in evaporating off the methyl alcohol. For this reason the urea has been determined in Experiments 4 and 6 in the blood before adding the ammonium carbonate, and the urea content of the fluid calculated from the result. For the same reason, the figures for the urea at the end of the experiment are in all probability minimal, for it is very likely that some ammonia is lost even there. Even assuming, however, that the total amount of nitrogen obtained after hydrolysis in determining the urea and ammonia together represents urea nitrogen alone, which cannot be so, for with the larger amounts the loss was only about 20 per cent, there is still not enough increase to equalize the loss in ammonia during the perfusion.

<sup>24</sup> *Loc. cit.*

crease in "rest nitrogen" in our experiments were due to washing out from the liver, it is hard to see why it should so nearly equal the ammonia unaccounted for.

In another experiment with ammonium carbonate, all but the ammonia determinations miscarried.

EXPERIMENT 7. Normal cat. Fluid: 87 cc. defibrinated cat blood and 103 mgms. ammonium carbonate in 5 cc. Ringer's solution. Perfused one hour.

	MILLIGRAMS PER 100 CC.	TOTAL AMOUNT IN MILLIGRAMS
	Ammonia nitrogen	Ammonia nitrogen
Before.....	23	21
After.....	7	7

The results of all the experiments with ammonium carbonate are given below in tabular form:

NUMBER OF EXP.	MILLIGRAMS PER 100 CC.							
	Before				After			
	Total n.p. nitrogen	Urea N	Ammonia N	Rest N	Total n.p. nitrogen	Urea N	Ammonia N	Rest N
1	62		22		54		7	
2	66		24		43		5	
3	62		14		60		4	
4	51	11	24	16	62	26	7	29
5	54		21		60		7	
6	52	11	28	13	48	15	7	26
7			23				7	

NUMBER OF EXP.	AMMONIA N IN PER CENT OF TOTAL N.P. N		UREA N IN PER CENT OF TOTAL N.P. N		PER CENT OF AMMONIA N LOST	PER CENT OF LOST AMMONIA N RECOVERED AS UREA
	Before	After	Before	After		
1	35	13			63	
2	36	12			67	
3	23	7			70	
4	47	11	22	42	77	56
5	39	12			69	
6	54	15	21	31	72	26

Therefore, from 63 to 77 per cent of the added ammonia disappeared, and of this only a part was recovered as urea.

## II. *Glycocoll*.<sup>35</sup>

EXPERIMENT 8. Normal rabbit. Fluid: 46 cc. rabbit serum, 36 cc. defibrinated rabbit blood, 23 cc. Ringer's solution and 183.8 mgms. glycocoll (34.3 mgms. nitrogen). Fifty minutes, twenty times. In, 105 cc. Out, 102 cc.

	MILLIGRAMS PER 100 CC.		TOTAL AMOUNT IN MILLIGRAMS	
	Total non-protein nitrogen	Urea and ammonia nitrogen	Total non-protein nitrogen	Urea and ammonia N.
Before.....	76	20	80	21
After.....	76	19	77	19

EXPERIMENT 9. Normal rabbit. Fluid: 31 cc. rabbit serum, 54 cc. defibrinated rabbit blood, 15 cc. Ringer's solution and 122.3 mgms. glycocoll (22.8 mgms. nitrogen). Sixty-one minutes. Thirty times. In, 100 cc. Out, 115 cc.

	MILLIGRAMS PER 100 CC.		TOTAL AMOUNT IN MILLIGRAMS	
	Total non-protein nitrogen	Urea and ammonia nitrogen	Total non-protein nitrogen	Urea and ammonia N.
Before.....	70	22	70	22
After.....	60	17	69	20

In the next two experiments, with cats, there appears to have been a certain amount of nitrogen washed out of the liver.

EXPERIMENT 10. Normal cat. Liver somewhat fatty. Starved twenty-four hours after meat régime. Fluid: 44.5 cc. defibrinated cat blood, 58 cc. Ringer's solution and 188.5 mgms. glycocoll (35.2 mgms. nitrogen). One hour, eighteen times. In, 102 cc. Out, 122 cc.

	MILLIGRAMS PER 100 CC.		TOTAL AMOUNT IN MILLIGRAMS	
	Total non-protein nitrogen	Urea and ammonia nitrogen	Total non-protein nitrogen	Urea and ammonia N.
Before.....	62	10	63	10
After.....	65	9	79	11

<sup>35</sup> Kahlbaum's glycocoll was used in all cases.

EXPERIMENT 11. Normal cat. Fluid: 56 cc. defibrinated cat blood, 19 cc. Ringer's solution and 188.7 mgms. glycocoll (35.2 mgms. nitrogen) in 5 cc. water. One hour. Thirty-one times. In, 80 cc. Out, 105 cc.

	MILLIGRAMS PER 100 CC.		TOTAL AMOUNT IN MILLIGRAMS	
	Total non-protein nitrogen	Urea and ammonia nitrogen	Total non-protein nitrogen	Urea and ammonia N.
Before.....	72	17	58	14
After.....	66	15	69	15

In the fluid at the end of the perfusion were found 1.9 milligrams of ammonia nitrogen, making the total urea nitrogen only 13 mgm. A comparison of this result with the normal amount of ammonia nitrogen in cat's blood found by Folin and Denis (about 0.07 mgm. per 100 cc.) demonstrated that there is formation of ammonia during the course of an hour's perfusion. The next experiment (12) shows the same thing. As stated earlier, in no case have we been able to get enough ammonia from 5 cc. of normal blood to be anywhere nearly readable in the Duboscq colorimeter as ordinarily used, *i.e.*, without the polariscope tube and iris diaphragm used by Folin and Denis.

EXPERIMENT 12 Normal cat. Starved twenty-four hours. Fluid: 76 cc. blood and 179.5 mgms. glycocoll (33.5 mgms. nitrogen) in 4 cc. water. Total time, three hours. Analysis of fluid after one hour as well as at end. In, 80 cc. Out, in one hour, 105 cc.; in, three hours, 84 cc.

	MILLIGRAMS PER 100 CC.				TOTAL AMOUNT IN MILLIGRAMS			
	Total n.p. N	Urea and amm. N	Urea N	Ammonia N	Total n.p. N	Urea and amm. N	Urea N	Ammonia N
Before.....	75	12.0			60	9.6		
After 1 hour..	58	10.0			removed 61	10.5		
					returned 58	10.0		
After 3 hours.	69	13.8	11.6	2.2	58	11.6	9.8	1.8

5 cc. of the 105 removed at the end of one hour were used for analysis, the other 100 cc. returned to the apparatus.

In the following experiment the outflow of fluid from the liver was obstructed early in the perfusion, causing the liver to swell permanently. Therefore, the recovery was only partial. Ringer's

solution had to be added to make the volume large enough to continue the experiment.

EXPERIMENT 13. Normal cat. Starved twenty-four hours after meat diet. Fluid: 81 cc. defibrinated cat blood and 173 mgms. glycocoll (32.3 mgms. nitrogen) in 9 cc. water. Forty minutes, thirty times. In, 90 cc. Out, 97 cc.

	MILLIGRAMS PER 100 CC.		TOTAL AMOUNT IN MILLIGRAMS		UREA + NH <sub>3</sub> -N TOTAL N. P. N <i>per cent</i>
	Total non-protein nitrogen	Urea and ammonia nitrogen	Total non-protein nitrogen	Urea and ammonia N	
Before . . . .	88	23	78	20	26
After . . . . .	50	11	48	11	23

It is seen that in none of the experiments with glycocoll is there any suggestion of urea formation (not even in Experiments 10 and 11, in which a considerable amount of nitrogen was washed out of the liver), although precisely similar experiments with ammonium carbonate demonstrate the ability of the surviving liver under these conditions to metabolize considerable amounts of ammonia, and, in the two experiments in which data are available (4 and 6), to form urea, or some nitrogenous substance not blown over by the air-current, but hydrolyzed at 150°C. under the conditions of the determination. The concentrations of glycocoll added have been about those used by Salaskin.

In the introductory part of this paper we have offered a number of possible explanations of this difference in results. It is not at all likely that the difference in the animals used would lead to so different results.

We by no means wish to argue, from the results obtained, that urea formation from amino-acids does not occur in the liver at all, for such a conclusion would be quite unjustifiable on the basis of the above data. We do believe, however, that it is extremely doubtful that such a process has ever been demonstrated.

The only amino-acid used by us has been glycocoll, but inasmuch as there is no evidence for urea formation from other amino-acids in the liver that is not subject to the same criticism as in the case of glycocoll, we feel justified in making the statement more general.

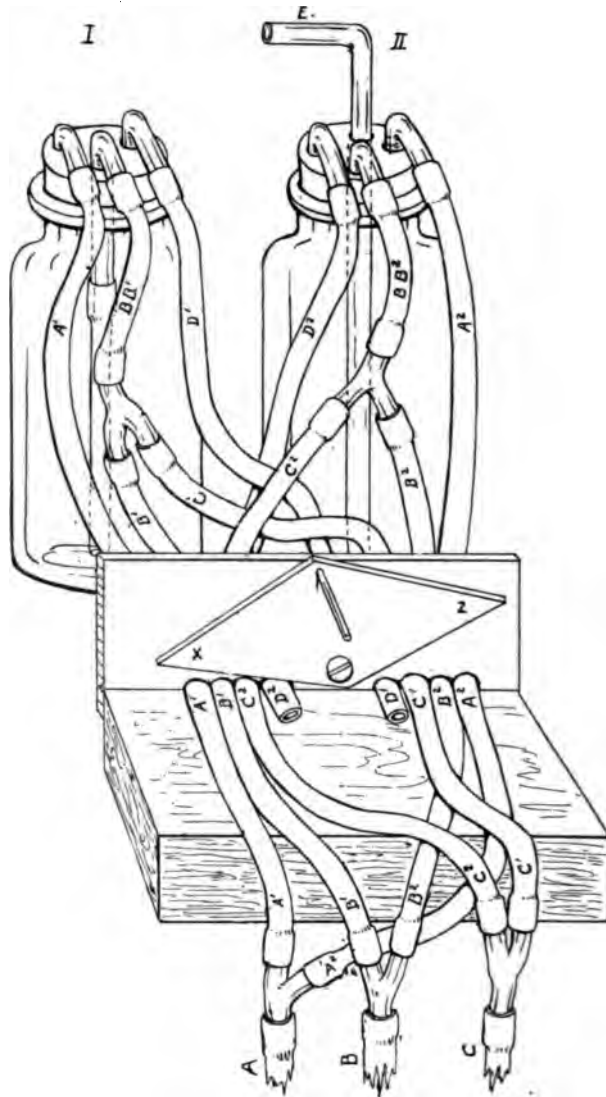
## SUMMARY AND CONCLUSIONS.

1. The surviving liver is capable of destroying ammonia perfused through it in the form of ammonium carbonate, and of converting it partially into urea. The entire amount of ammonia changed, however, does not have this fate. How much, if any, of it undergoes synthesis to amino-acids has not been determined. It is doubtful whether the binding of ammonia as such by the liver cells is of much significance in the protective influence of the organ, as indicated by the lack of variation in total non-protein nitrogen content of the fluid during the experiment.

2. The perfusion of the liver of the cat or the rabbit with homologous defibrinated blood containing as much as 44 mgm. of nitrogen as glycocoll per 100 cc. does not lead to any increase in the amount of urea in the fluid used.

3. The formation of urea from amino-acids by the liver is not conclusively demonstrated. There is no incontestable ground for the assumption that the liver is a special site for such a process.

We are greatly indebted to Professor Otto Folin for valuable advice received at various times during the course of this work.





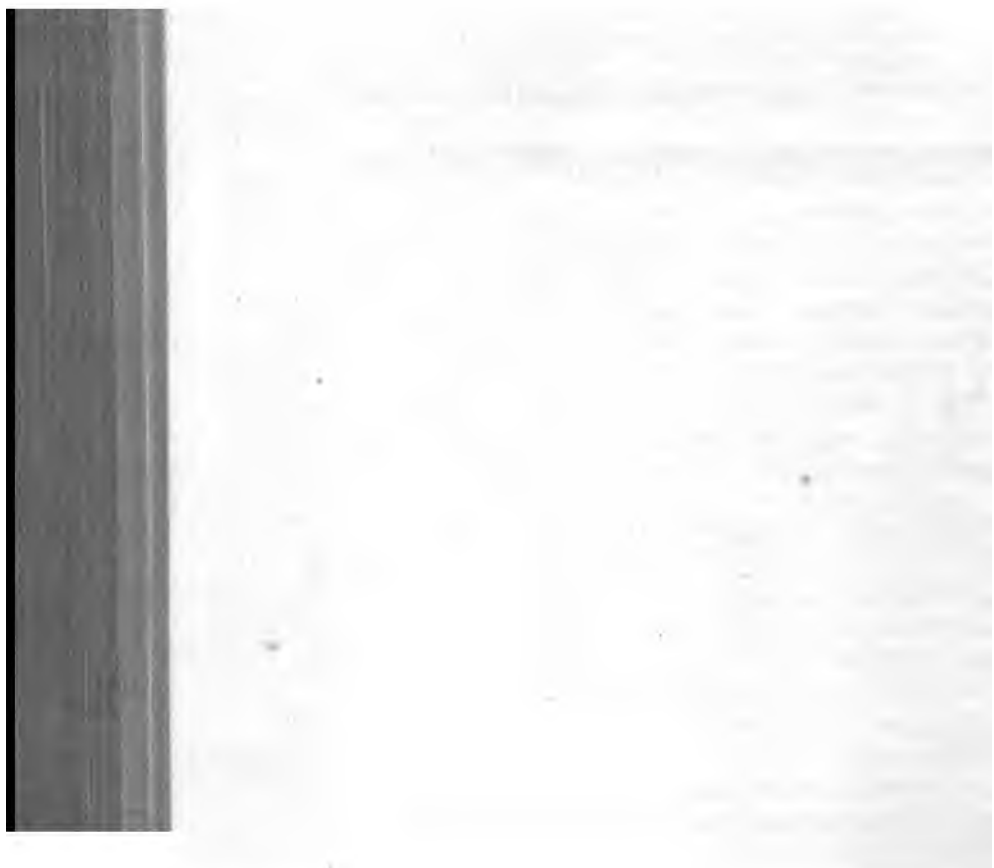
## EXPLANATION OF DIAGRAM OF PERFUSION BOTTLES.

Tube *A* is for inlet of air pressure, passing through *A*<sup>1</sup> or *A*<sup>2</sup> to the bottles. Fluid passes through *B*<sup>1</sup> or *B*<sup>2</sup> to *B*. Fluid returns through *C* to *C*<sup>1</sup> or *C*<sup>2</sup>, and thence to bottles. Air is released from bottles through tubes *D*<sup>1</sup> or *D*<sup>2</sup>. When the rocker valve is closed on *Z* side, the air pressure reaches bottle *I*, tube *D*<sup>1</sup> being closed on *Z* side of throttle; fluid passes through tube *BB*<sup>1</sup> to *B*<sup>1</sup>, the arm of the Y-tube connecting with *C*<sup>1</sup> not permitting passage of fluid because of closure by *Z* side of rocker. Fluid passes thence through *B*, through water coil, liver and returns to bottle *II* by way of *C* and *C*<sup>2</sup>, the latter being open on *X* side of throttle; fluid passes into bottle *II* because air is allowed to escape through *D*<sup>2</sup>.

*D*<sup>1</sup> and *D*<sup>2</sup> were frequently connected with a Y-tube and the exhaust air passes through  $\frac{N}{10}$  HCl, but not enough ammonia was detected with Nessler's reagent to affect the figures.

Tube *E* was kept closed by means of a clamp, but when bottle *II* was filling, air freed from ammonia was forced into the bottle so as to aerate the fluid.

With reversal of the rocker, the process was reversed but without noticeable interruption to the outflow through *B* and inflow through *C*, although extremely careful attention had to be given water coil and thermometer at this time.



## THE SATURATED FATTY ACID OF KEPHALIN.

By P. A. LEVENE AND C. J. WEST.

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(Received for publication, October 22, 1913.)

The work on the composition of kephalin has made little progress from 1909 until the last few months. Last winter we had in our possession a considerable quantity of the ether-soluble fraction of brain lipoids and hence employed it for preparation of kephalin. The preparation was carried out following the process employed by previous workers.<sup>1</sup> The purification was accomplished by repeatedly dissolving the crude substance in petroleic ether (boiling point, 40–50°C.) and precipitating it with alcohol. This operation was repeated about ten times, until the final product had on drying a light straw-yellow color. The ethereal solution was allowed to flow into the alcohol in a very slow stream, mechanical stirring being employed during the precipitation and at least about one-half hour after the completion of the precipitation. The product was then suspended in acetone, which was allowed to act on it under constant stirring. The final product was a dry, light powder.

At the close of last season the work was completed only so far as it concerned the saturated fatty acids obtainable on hydrolysis of kephalin. There existed conflicting statements regarding their nature, Cousin<sup>2</sup> having isolated only stearic acid, while Fränkel and Neubauer claimed the presence in kephalin of both stearic and palmitic acids.

Four samples of kephalin were analyzed and in all of them was found on hydrolysis only one saturated fatty acid, namely, stearic

<sup>1</sup> Falk: *Biochem. Zeitschr.*, xiii, p. 153, 1908; xvi, p. 187, 1909; Fränkel and Neubauer: *ibid.*, xxi, p. 321, 1909; Fränkel and Dimitz: *ibid.*, xxi, p. 327, 1909; Parnas: *ibid.*, xxii, p. 411, 1909.

<sup>2</sup> Cousin: *Jour. d. pharm. et d. chim.*, xxiv, p. 101, 1906; xxv, p. 177, 1907.

acid. The publication of this result was planned to resume the further study of the in course of the present season. However in course of the summer a very important publication by his co-workers<sup>3</sup> on the nature of the nitrogenous substance, cephalin, and in the last number of the *Journal of Biological Chemistry*, another publication, also by Parnas,<sup>5</sup> on the nature of cephalin, in which the writer arrives at the conclusion that stearic acid is the only saturated fatty acid of cephalin.

We therefore concluded to present the results of our work not only because they strengthen the results of the other workers but also for the reason that in four out of five cases the separation of the saturated from unsaturated fatty acids was carried out by a process different from the one used by the previous workers.

The separation was based on the difference in solubility of the ethyl esters of the saturated and unsaturated fatty acids. The saturated acid precipitates out of the solution, while the unsaturated still remains in solution. The results obtained with the saturated acid is perhaps not absolutely neat and convenient. The results obtained with the unsaturated acid of cephalin were corroborated by the results of the hydrolysis by means of a barium hydroxide solution.

#### EXPERIMENTAL PART

The cephalin used in this investigation was obtained by dissolving it in 40–50° petroleum ether and then precipitating it with stirring into alcohol. Two different methods were used for nitrogen and phosphorus.

<sup>3</sup> Baumann: *Biochem. Zeitschr.*, liv, p. 30, 1913.

<sup>4</sup> Parnas: *Ibid.*, lvi, p. 17, 1913.

<sup>5</sup> Parnas, referring to the article by one of us, *loc. cit.*, xv, p. 153, attributes to the writer the statement that the substance obtained on hydrolysis of a lipoid by means of alkaline phosphoric acid. This was an error on the part of Parnas. It is clearly stated in the article referred to that the first product obtained on the hydrolysis of the lipoid was stearic acid.

- I. 0.2988 gram substance gave 0.0430 gram  $\text{Mg}_2\text{P}_2\text{O}_7$ .  
 0.2004 gram substance required 2.15 cc.  $\frac{\text{N}}{16}$  HCl (Kjeldahl).  
 II. 0.3058 gram substance gave 0.0440 gram  $\text{Mg}_2\text{P}_2\text{O}_7$ .  
 0.4152 gram substance required 4.8 cc.  $\frac{\text{N}}{16}$  HCl (Kjeldahl).

	Found:	
	I	II
P.....	4.01	4.01
N.....	1.50	1.62

#### *Hydrolysis with alcoholic HCl.*

Fifty grams of kephalin were heated with 600 cc. methyl alcohol and 20 cc. concentrated sulphuric acid ten hours under a reflux and in an atmosphere of carbon dioxide. At the end of the heating there was a slight mineral residue. The colored solution was decanted from this residue and cooled in the ice box over night. The precipitate which formed was filtered off and recrystallized from acetone. It melted at 37–38°. Methyl stearate melts at 38°. The ester was hydrolyzed by heating with alcoholic potassium hydroxide, the soaps decomposed by warm hydrochloric acid, washed free from acid and recrystallized from acetone. The first fraction was analyzed (I), then further purified by changing into the lead salt and decomposing with hydrogen sulphide in toluene; this was then recrystallized from acetone and the last trace of solvent removed by melting. The mother liquor from (I) was concentrated and the acid which separated analyzed (II). Four different hydrolyses were carried out with similar results. The four samples showed m.p. of 69°, 69°, 68–69°, 69°, and gave no depression when mixed with a sample of Kahlbaum's stearic acid, purified through the lead salt.

#### *Analysis of the acid.*

- I. 0.1280 gram of the substance gave 0.3557 gram  $\text{CO}_2$  and 0.1492 gram  $\text{H}_2\text{O}$ .  
 II. 0.1388 gram of the substance gave 0.3860 gram  $\text{CO}_2$  and 0.1576 gram  $\text{H}_2\text{O}$ .

	Calculated for	Found:	
	$\text{C}_{18}\text{H}_{35}\text{O}_2$	I	II
C.....	76.00	75.79	75.84
N.....	12.70	13.05	12.71

#### *Molecular weight estimations.*

Samples from four different hydrolyses were used, all of which were purified through the lead salt.

## 422 The Saturated Fatty Acid

- I. 1 gram of the acid dissolved in a mixture of ether and benzene, when titrated with  $\frac{N}{10}$  alkali, using phenolphthalein as indicator, required 34.8 cc.  $\frac{N}{10}$  NaOH for neutralization.
- II. 1 gram of the acid, as above, required 34.6 cc.  $\frac{N}{10}$  NaOH.
- III. 1 gram of the acid, as above, required 34.6 cc.  $\frac{N}{10}$  NaOH.
- IV. 1 gram of the acid, as above, required 34.6 cc.  $\frac{N}{10}$  NaOH.

	Calculated for	
	$C_{18}H_{36}O_2$	1
M. W. ....	286	287

### *Aqueous barium hydroxide*

A fifth sample of the same kephalin was hydrolyzed with aqueous barium hydrate in an autoclave. The mixture of barium salts was filtered and the barium removed by repeated extraction with ether. The ether was liberated with warm dilute hydrochloric acid, the lead salt, decomposed with hydrogen sulphide from acetone. The acid thus obtained melted at 68–69°C. with pure stearic acid, it melted at 68–69°C. No other saturated fatty acid could be found.

1 gram of the acid, as above, required 34.6 cc.  $\frac{N}{10}$  NaOH.

M. W. ....

## THE INFLUENCE OF BUTTER-FAT ON GROWTH.<sup>1</sup>

By THOMAS B. OSBORNE AND LAFAYETTE B. MENDEL,

With the Coöperation of EDNA L. FERRY and ALFRED J. WAKEMAN.

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and the Sheffield Laboratory of Physiological Chemistry  
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(Received for publication, November 4, 1913.)

We have recently pointed out<sup>2</sup> that while young rats grow for a time at a normal rate on the "protein-free milk" diet used in our earlier experiments, they sooner or later cease to grow, so that they rarely attain more than two-thirds of the weight normal for fully grown rats receiving a diet chiefly composed of milk and lard. Furthermore, we showed that rats which had ceased to grow and were declining on a "protein-free milk" diet, at once recovered and resumed a normal rate of growth when a part of the lard in their food was replaced by a quantity of unsalted butter corresponding to that in the milk-food. The striking way in which butter, thus supplied, influenced the growth of these young rats made it evident that it furnishes some substance which exerts a marked influence on growth.

These observations have since been verified by numerous additional experiments and an attempt has been made to determine with which of the components of the butter this growth-promoting power is associated. As is well known, butter consists of about 82-83 per cent of the glycerides of numerous fatty acids, about 15 per cent of water containing each of the soluble constituents of milk, and from 1 to 2 per cent of solid matter, consisting chiefly of cellular débris from the mammary glands, bacteria, calcium

<sup>1</sup> The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

<sup>2</sup> Osborne and Mendel: The Relation of Growth to the Chemical Constituents of the Diet, this *Journal*, xv, pp. 311-326, 1913.

phosphate, particles of casein, and accidental impurities introduced during the process of making the butter.

In view of the possibility that even an extremely minute quantity of some substance might exert the favorable influence on growth observed in all of our experiments, we separated the butter into three parts, namely the fatty substances, the insoluble solid elements, and the aqueous solution containing lactose, soluble inorganic salts and other soluble components of the milk; and by feeding trials found that the growth-promoting factor was contained in the fat fraction. Further consideration of the two other fractions is therefore unnecessary.

The butter-fat used in the analytical, as well as the feeding experiments here described, was prepared as follows. The butter was melted by heating in a flask immersed in a bath of water not exceeding 45°, and centrifugated for about an hour at a high speed. The melted butter was thus separated into a layer of perfectly clear fat, an opalescent aqueous layer, and a deposit of white solid matter. The clear fat was sucked off with care, and thus separated from all of the other parts of the butter. By this method the use of all solvents was avoided, and any substances which might have been dissolved thereby from the other parts of the butter were excluded.

So much has been written about the significance of phosphatides (lecithin, etc.) in various biological phenomena, and in growth among others, that a careful analysis of large quantities of the butter-fat was instituted to detect the presence of members of this group of so-called lipoids. The butter-fat prepared as described was found to be entirely free from nitrogen and phosphorus and was devoid of any ash-yielding, or water-soluble, components. The absence of phosphatides from the product corresponds with the recent statement of Njegovan<sup>3</sup> who concludes that milk contains no lecithin whatever, and suggests that the contradictory claims of other investigators are attributable to inadequate methods of analysis.

Butter-fat, thus prepared, has proved to be quite as effective as butter or milk in promoting the recovery and renewed growth of animals which have ceased, or failed, to grow on the natural

<sup>3</sup> Njegovan: *Biochem. Zeitschr.*, liv, p. 78, 1913.



"protein-free milk" dietaries in which lard furnished the fat component. Our previously published experiments<sup>4</sup> in which similar recoveries were made when *butter* replaced a part of the lard of diets containing the "artificial protein-free milk" are complicated by the fact that butter contains about 15 per cent of buttermilk, and hence the improvement shown by such experiments might be attributed to some constituent of the buttermilk. Experiments with "artificial protein-free milk" and butter-fat are in progress and it is hoped by these to learn whether or not accessory substances other than those contained in the butter-fat are necessary for growth. These experiments are not yet completed.

Charts showing the body-weights of growing rats that had begun to decline on our "protein-free milk" food mixtures, and were supplied with foods having butter or butter-fat introduced,<sup>5</sup> are appended (see Charts I, II, and III). The composition of the mixtures was as follows:<sup>6</sup>

	Butter foods per cent	Butter-fat foods per cent
Protein.....	18	18
Starch.....	26	26
Protein-free milk.....	28	28
Lard.....	10	10
Butter-fat.....	15.3	18
Buttermilk, etc.....	2.7	0

The efficiency of the butter-fat, or some component thereof, in specifically promoting growth is further shown in another way. As has already been pointed out, when very young rats are placed on a mixture of purified protein, lard, starch, and "protein-free milk," prepared in imitation of the gross composition of the highly successful milk-food, they show a varying capacity to grow. In some cases growth has stopped after sixty days; other animals have continued to grow for one hundred days or more. But in the growth experiments there has always been an inevitable ulti-

<sup>4</sup> This *Journal*, xv, p. 326, 1913.

<sup>5</sup> For similar recoveries induced by milk-food, see Charts II and III, this *Journal*, xv, pp. 321-322, 1913.

<sup>6</sup> For details regarding the milk-foods used in our experiments, and other comparisons, see Osborne and Mendel: this *Journal*, xv, p. 318, 1913.

mate inhibition of growth, and nutritive decline, connected with some diet factor. Very few of our rats grew after they were 140 days old, at which age two-thirds of the normal growth of the male, and three-fourths of that of the female is usually made. We have accordingly attempted to learn whether the continued exhibition of butter-fat from very early in the growth period would enable the animals to attain their normal maximum weight and thereby avert the invariable failure which hitherto was met with sooner or later. If it be assumed that in these earlier experiences the substance, or substances, essential for growth, and supplied inadequately, or not at all, in the artificially prepared diets, is furnished by a reserve stored in the cells of the young animals, this must be exhausted after a time, and lead to failure of growth. If, however, a growth-promoting substance is present in the butter-fat, and the latter is supplied in abundance, growth ought to continue to its logical conclusion, in the absence of other inhibitory factors.

Experiments to test this were begun with young rats whose diet from an early period consisted of food of the following composition:

	per cent
Purified protein.....	18
Starch.....	26
Protein-free milk.....	28
Lard.....	10
Butter-fat.....	18

In harmony with the theory outlined above, the outcome of these newer feeding trials already demonstrates a far more successful continuance of growth on the foods containing butter-fat than on any other artificial diet-mixture (except milk-food) hitherto tested. An illustrative chart (IV) is appended.

In further corroboration of the efficiency of the butter-fat in promoting growth, and particularly after a previous decline, we may cite additional experiments involving the use of centrifugated, or so-called skimmed milk as the basis of the ration. A food paste, intended to resemble our successful milk-food, was prepared by the use of dried centrifugated milk.<sup>7</sup> The product used

<sup>7</sup>Like the whole milk this was supplied to us in powdered form by the Merrell-Soule Company of Syracuse, N. Y.

contains only 1.18 per cent of fats. The food had the following composition:

	per cent
Dried centrifugated milk.....	44
Starch.....	28
Lard.....	28

This food, although not entirely free from milk-fat, contained only 0.52 per cent, *i.e.*, less than one twenty-fifth as much as the whole milk-food used for our earlier experiments. Although rats have grown normally upon our whole milk-food for more than a year, the centrifugated milk-food has in every case failed to prove adequate for a comparable period. It is true that the centrifugated milk-food has been more satisfactory as a growth ration than our "protein-free milk" foods, *i.e.*, the moment of ultimate failure to grow or to decline has been postponed longer. This may well be due to the small quantities of the essential butter-fat still present in the commercial product used by us. However, the substitution of a part of the lard in the food-pastes by an amount of butter equivalent to that naturally present in our milk-foods brought prompt recovery, as exemplified in the appended chart V.

The outcome of these experiments clearly indicates that the growth-promoting substance of the milk is to be found in the butter-fat fraction thereof; for the two rations here illustrated and characterized by either nutritive failure or success, differ in respect to the fat component only. The influence of heating and other processes involved in the preparation of milk for food were also incidentally investigated. These studies, though far from completed, have given no evidence, in so far as nutrient efficiency is concerned, of a damage to the centrifugated milk by vigorous sterilization. We hope to return to this question in a later communication.

The experiments recently reported by McCollum and Davis,<sup>8</sup> who added an ether extract of butter or eggs to artificially prepared food-mixtures, are in accord with the results which we have obtained by feeding butter, or butter-fat, to rats which were declining after a period of growth on a diet of isolated food-sub-

<sup>8</sup> Cf. McCollum and Davis: The Necessity of Certain Lipins in the Diet during Growth, this *Journal*, xv, p. 167, 1913.

stances. In none of their published records was the gain as rapid as in most of ours, nor was the rate of gain after reaching the previous maximum weight as high as on the butter-fat-free diet earlier supplied. In the case they report, the gain made by their rats after the previous maximum weight was 40, 25, 4 per cent, the last of the latter gain being made after the rats had reached the young. The time during which the rats were maintaining the ether extract was 5, 9, 12, and 15 days, in the case did the animal reach a weight normal to that of the case the data furnished by McCollum and Davis, and that butter-fat has a marked influence on the growth means prove that butter-fat contains something essential to the metabolism of growth, apart from that which is added butter-fat may have simply supplied the so-called vitamins, which Funk has shown are essential for life, and thereby enabled the animals to live on food thus made adequate for maintenance. It is shown that rats can be maintained for long periods on the diet of McCollum and Davis used no final conclusion respecting the above question.

By numerous experiments we have shown that rats can be maintained on our "protein-free milk diet" for a year, and that young rats on similar diet, though it is inadequate for growth can be maintained on these foods consequently supply all that is essential for life alone. Since growth ceases on these foods for a short time, and is at once resumed and continues through the entire period of normal growth when a pair of rats is fed by butter-fat, it is almost certain that butter-fat is something essential for growth in addition to that which is required for maintenance. This recovery and regrowth is attributed to something which distinguishes butter-fat from other fatty acids, for not only do lard and olive oil promote growth, but young rats grow on these foods when all of the lard is replaced by ether-soluble substances are present in the

<sup>9</sup> McCollum and Davis: *loc. cit.*

<sup>10</sup> Cf. Osborne and Mendel: Feeding Experiments with Mixture, this *Journal*, xii, pp. 81-89, 1912.

It thus appears improbable that glycerides of the fatty acids ordinarily present in foods are responsible for the promotion of the growth observed when butter-fat replaces lard in the diet of rats which have ceased to grow. Lecithin and other phosphorus- or nitrogen-containing substances are excluded by the absence of phosphorus and nitrogen from our butter-fat; and cholesterol by the fact that even more of this substance has been obtained from lard than from butter.<sup>11</sup>

So far as our experience has shown, the addition of butter-fat to our natural "protein-free milk" foods gives them an efficiency quite comparable with that of our milk-food in promoting recovery and the completion of growth. The exact chemical differences between the adequate butter-fat and the inadequate lard (which determine success and failure respectively in the food-mixtures employed) are far from being satisfactorily known. Chemical examination of the butter-fat indicates that the effective component is not a phosphatide or any inorganic substance, inasmuch as nitrogen, phosphorus and ash are lacking in the product employed. It is suggestive to note that in the one case (lard) we are dealing essentially with a fat-mixture deposited in storage depots of the animal organism; in the other, the butter-fat represents the product of metabolic activity and synthesis on the part of the cells of the mammary gland. What, if anything, this distinction between cellular product and reserve fat may mean physiologically, remains to be investigated.

The researches which have been devoted in recent years to certain diseases, notably beri-beri, have made it more than probable that there are conditions of nutrition during which certain essential, but, as yet, unknown substances must be supplied in the diet if nutritive disaster is to be avoided. These substances apparently do not belong to the category of the ordinary nutrients, and do not fulfil their physiological mission because of the energy which they supply. Funk has proposed the name *vitamine* for the type of substance thus represented.<sup>12</sup>

Without minimizing the importance of the new field of research and the new viewpoints in nutrition which are presented by these

<sup>11</sup> Cf. McCollum and Davis: *loc. cit.*, p. 174, who fed cholesterol to rats.

<sup>12</sup> The literature on the subject has been reviewed by Funk: Ueber die physiologische Bedeutung gewisser bisher unbekannter Nahrungsbestandteile der Vitamine, *Ergeb. d. Physiol.*, xiii, p. 125, 1913.

recent findings, we may nevertheless hesitate to accept the extreme generalizations which have already been proposed on the basis of the evidence obtained largely from the investigation of pathological conditions. The statement, for example, that a "tadellose Nahrung" may prove entirely inadequate unless "vitamines" are present, at once suggests a series of questions bearing on what is included in the new term. It is still rather early to generalize on the rôle of accessory "vitamines" when the ideal conditions in respect to the familiar fundamental nutrients and inorganic salts adequate for prolonged maintenance are not completely solved. Speculation is quite justifiable in so far as it directs attention to a new phase that needs to be taken into account.

Funk has expressed the belief that the substance which promotes growth and must be present in order to avert the cessation of growth, which we have described to occur after a certain period of successful growth on our earlier dietaries, is either identical with, or analogous to, the "vitamine" which plays the rôle of an antiscorbutic substance. For this we can as yet find no compelling evidence. Certainly the nitrogen-free butter-fat, so successful in remedying our growth failures, contains no substance chemically related to the nitrogenous products which have lately been credited with this unique physiological efficiency.<sup>13</sup> Furthermore it

<sup>13</sup> Cf. Funk: Ueber die physiologische Bedeutung gewisser bisher unbekannter Nahrungsbestandteile der Vitamine, *Ergeb. d. Physiol.*, xiii, p. 130 *et seq.*, 1913. In reviewing our earlier published experiments Funk has erroneously assumed that we secured *completed* growth with the diets in which the butter component was not yet employed. It is true that the increments in weight were in some cases very noteworthy; but in every instance cessation ultimately ensued before the completion of the normal progress of the growth or subsequent maintenance. We have never denied the necessity of a growth-promoting food accessory in accord with the claim of Hopkins; and recently we pointed out that the successful partial completion of growth, such as has been obtained in our experiments, may well have been due to a store of the essential compound in the body of the experimental animals at the beginning of the trials. It is by no means necessary to assume with Funk that small quantities of these accessory substances were inadvertently left in our food preparations owing to insufficient extraction with alcohol.

Furthermore we cannot agree with Funk that the rat is not well adapted to experiments on the physiology of growth. The superiority of this animal has been pointed out by us elsewhere (cf. this *Journal*, xiii, p. 233,

is well to bear in mind that it is not improbable that the anti-neuritic and antiscorbutic constituents of foods are not identical with the substances alleged to assist in maintaining body-weight.<sup>14</sup> Funk<sup>15</sup> has lately asserted that the simultaneous administration of at least two substances is necessary to produce the curative effect obtained in his previous experiments with the "vitamine" fraction from rice-bran or yeast. Voegtlin and Towles<sup>16</sup> have noted that extracts of autolyzed spinal cord may be antineuritic, yet be unable to reestablish normal metabolism, i.e., restore body-weight.

Butter-fat has shown a further interesting nutritive superiority over lard. At certain periods of the year, particularly in summer months, we have frequently failed to secure satisfactory growth on the dietaries which proved adequate during the usual period of sixty to one hundred days at other seasons. Occasionally young rats in the stock colony have exhibited a similar "epidemic" of poor growth at the same season. The failures are, however, not common to rats fed on the milk-food; and we have lately observed that the seasonal failure is also averted by the addition of butter-fat to the usual "protein-free milk" food-mixtures.<sup>17</sup> Again, another type of nutritive deficiency exemplified in a form of infectious eye disease prevalent in animals inappropriately fed<sup>18</sup> is speedily alleviated by the introduction of butter-fat into the experimental rations.

The chemical character of the unique "accessory substance" in butter-fat must be investigated in detail and its possible pres-

1912) and is also apparently recognized by both Donaldson and McCollum and their coworkers. We have found rats to be responsive to changes in diet; and we count it no disadvantage that the experiments must be continued over sufficient time to exclude minor incidental fluctuations.

<sup>14</sup> Cf. Cooper: *Journ. of Hygiene*, xii, p. 433, 1912.

<sup>15</sup> Funk: *Ergeb. d. Physiol.*, xiii, p. 547, 1913; *Brit. Med. Journ.*, April 19, 1913; *Journ. of Physiol.*, xlvi, p. 173, 1913.

<sup>16</sup> Voegtlin and Towles: *Journ. of Exp. Pharmacol.*, v, p. 67, 1913.

<sup>17</sup> These summer failures in growth have been reported to us by colleagues to occur likewise in other institutes.

<sup>18</sup> Cf. Knapp: Experimenteller Beitrag zur Ernährung von Ratten mit künstlicher Nahrung and zum Zusammenhang von Ernährungsstörungen mit Erkrankungen der Conjunctiva, *Zeitschr. f. exp. Path.*, v, pp. 147-170, 1908.

ence elsewhere determined. Experiments are already under way with varying proportions of butter-fat in the ration; but we have not thus far determined the necessary allowance. On the other hand, no amount of butter-fat will induce growth on certain dietaries in which the proportions and nature of the inorganic salts are inappropriate (as in our Salt mixture I),<sup>19</sup> or the quantity and character of the protein is inadequate. The "Bausteine" must not be overlooked in our enthusiasm for these newer features.

ADDENDUM. An investigation now under way to determine the possible efficiency of fats other than butter-fat in preventing decline on our protein-free milk-food and promoting growth in the way that butter does, has already indicated marked differences in fats from different sources. Egg yolk-fat, for example, appears to behave like butter-fat; some other oils have thus far proved no more efficient than lard. Such considerations make it evident that the comparative value of the natural fats employed in nutrition must be determined, as well as the individual rôle of the different proteins, carbohydrates, and mineral nutrients.

<sup>19</sup> See Osborne and Mendel: Carnegie Institution of Washington, Publication 156, pt. ii, p. 80.



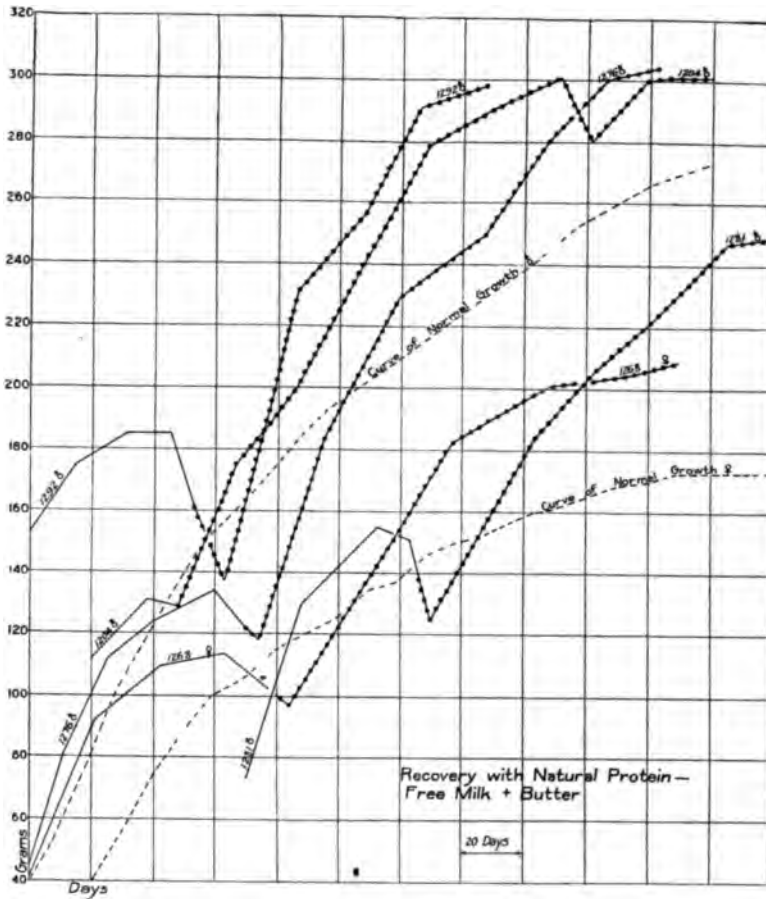


CHART I. Curves of body-weight of rats which have ceased to grow and have declined on foods containing the natural "protein-free milk," and have recovered when 18 per cent of unsalted *butter* replaced the same quantity of lard in the diet, as indicated by the interrupted lines (—o—o—o—). The proteins furnished in the different experiments were as follows: casein, Rats 1204, 1268, 1276, 1281, 1292; ovalbumin, Rats 1268, 1276.

The ordinates represent grams of body-weight, as indicated. The divisions of the abscissa represent 20-day periods.

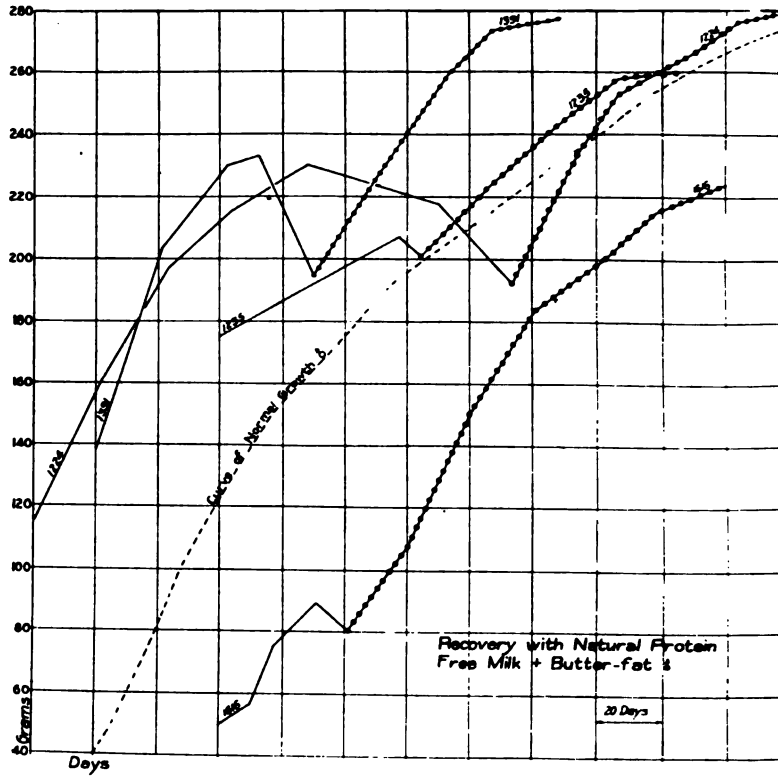


CHART II. Curves of body-weight of male rats which have ceased to grow and have declined on foods containing the natural "protein-free milk," and have recovered when 18 per cent of *butter-fat* replaced the same quantity of lard in the diet, as indicated by the interrupted lines (-o-o-o-o). The proteins furnished in the different experiments were as follows: casein, Rats 1224, 1235; edestin, Rat 1391; zein + casein, Rat 1616.

The ordinates represent grams of body-weight, as indicated. The divisions of the abscissa represent 20-day periods.

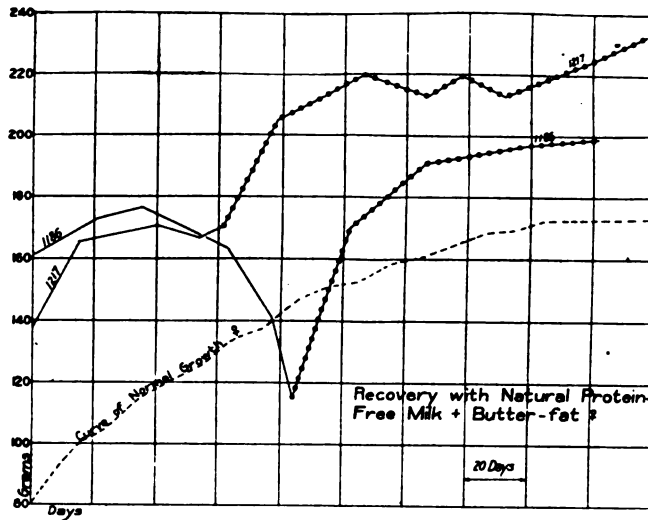


CHART III. Curves of body-weight of female rats which have ceased to grow and have declined on foods containing the natural "protein-free milk," and have recovered when 18 per cent of *butter-fat* replaced the same quantity of lard in the diet, as indicated by the interrupted lines (-o-o-o-o-). The proteins furnished in the different experiments were as follows: casein, Rat 1217; zein + lactalbumin, 1186.

The ordinates represent grams of body-weight, as indicated. The divisions of the abscissa represent 20-day periods.

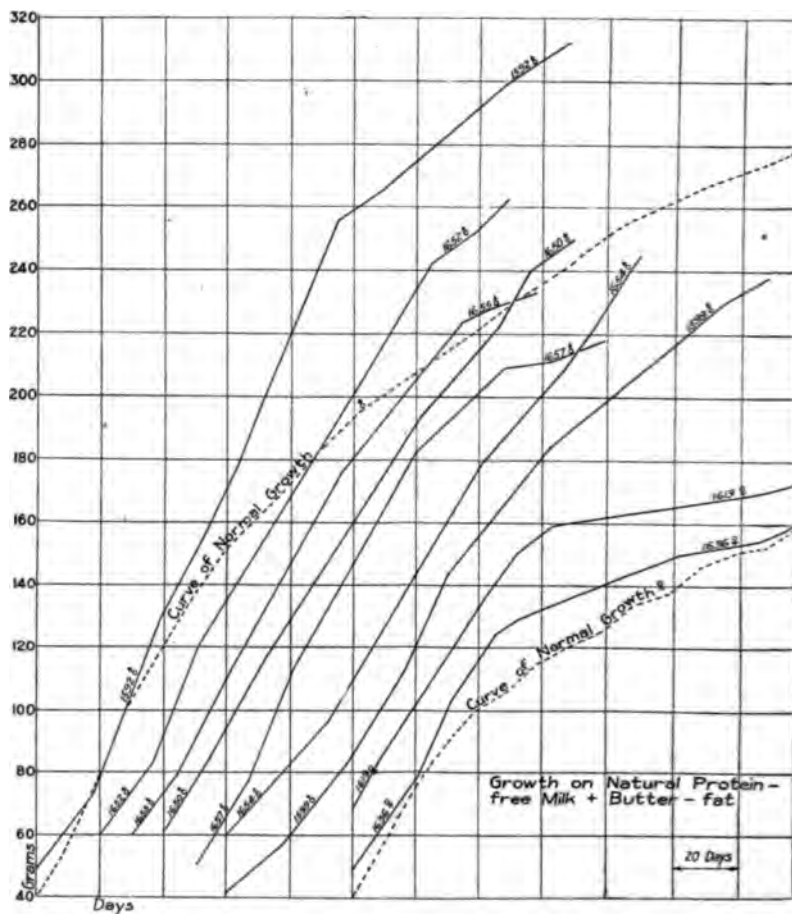


CHART IV. Typical curves showing prolonged normal growth of white rats on foods containing 18 per cent of *butter-fat*. The proteins furnished in the different experiments were as follows: casein, Rats 1592, 1599, 1619, 1636, 1652, 1655, 1657; edestin, Rats 1650, 1654.

The ordinates represent grams of body-weight, as indicated. The divisions of the abscissa represent 20-day periods.

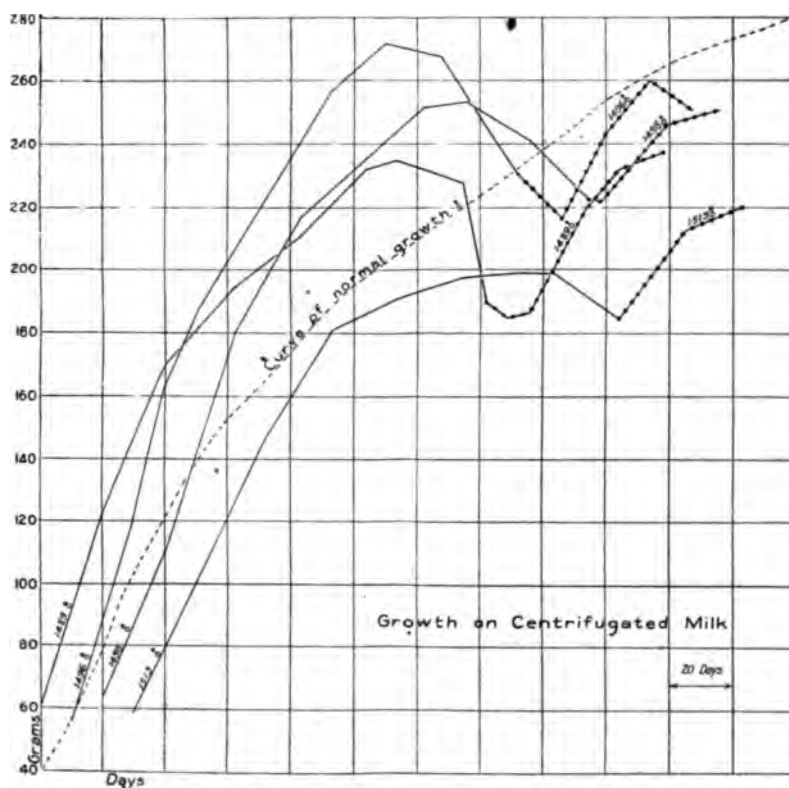


CHART V. Curves of the body-weight of rats which have ceased to grow and have declined on the *centrifugated-milk-food*, and have recovered when 18 per cent of *butter-fat* replaced the same quantity of lard in the diet.

The ordinates represent grams of body-weight, as indicated. The divisions of the abscissa represent 20-day periods.



## THE EFFECT OF FERMENTS AND OTHER SUBSTANCES ON THE GROWTH OF BURLEY TOBACCO.

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The growth of the tobacco plant during the first stage is very slow compared with some of the plants comprising the general farm crops. This is shown by the fact that it requires about two months or more in the seed bed to become of sufficient size for transplanting. In other words, it takes the young plant from the time the seed is sown until it reaches this stage, from one-half to three-fifths as long as it requires after transplanting to grow to maturity. Therefore, it can readily be understood that if some way could be found of shortening the first period of growth until the transplanting stage is reached, this would save the grower considerable time in looking after the seed beds as well as decreasing the chances of insects, fungi and bad weather from injuring and destroying the plants. Furthermore, in sections of the country where tobacco might be grown but for the short season, this would be a benefit in assisting to produce a crop. The grower realizes these difficulties and takes advantage of all the best conditions for obtaining the largest plants in the seed bed in the shortest time by locating it in virgin soil with the best exposure and applies fertilizers if necessary.

A great deal of work has been done in the past few years on the changes taking place in the germination and growth of seeds. It is now generally recognized that these changes depend primarily upon what are commonly called ferments or enzymes. The seed, as is well known, contains a certain amount of reserve food material which nourishes the seedling and enables it to grow until it can draw on the soil for its future supply. This stock of food in the seed may be in the form of carbohydrates, oils, fats, protein, etc., and the functions of the different enzymes are to act

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upon their particular part of this food supply and convert it into soluble form which can be utilized by the seedling.

F. A. Waugh<sup>1</sup> has shown that by soaking old seeds of some plants in different enzyme solutions, higher percentages of germination could be obtained than in the same seeds soaked in water. He worked with tomato, cucumber, radish and watermelon seeds of different ages, from five to twelve years old, and obtained increased germination in some which had been soaked in diastase solution, given in the following table:<sup>2</sup>

*Influence of enzymes upon germination.*

Description of seeds	Solution employed	Per cent germination
Tomato, 12 years old.....	Water.....	12
" " " ".....	Diastase.....	85
" " " ".....	Water.....	34
" " " ".....	Diastase.....	70
" " " ".....	Water.....	14
" " " ".....	Diastase.....	24
" 5 " ".....	Water.....	36
" " " ".....	Diastase.....	46
Cucumber 5 years old.....	Water.....	44
" " " ".....	Diastase.....	54
Radish, 6 years old.....	Water.....	46
" " " ".....	Diastase.....	66

The tomato seeds above were of different varieties and the plan was followed of soaking the seed in water and the ferment solution for the same time, then draining and transferring them to the germination apparatus.

The tentative conclusions reached by Waugh were:

- (1) In some cases the percentage of germination in seeds is greatly increased by soaking for several hours in a solution containing some active enzyme or enzymes.
- (2) The vigor of the young plantlets is often enhanced at the same time.
- (3) Within limits these beneficial effects increase with the strength of the enzyme solution.
- (4) Diastase, either from malt or from various commercial preparations, seems to be most useful.

<sup>1</sup> Tenth Annual Report, Vermont Agric. Exp. Station, 1896, p. 106.

<sup>2</sup> Taken from the Research Bulletin No. 22, p. 105, of the Wisconsin Agricultural Experiment Station.



(5) Tomato seeds seem to respond especially well to the action of enzymes, particularly to the action of diastase.

S. M. Babcock<sup>3</sup> has found in a similar experiment that corn, less than 50 per cent of which germinated when the seeds were soaked in water, all germinated when they were soaked in commercial diastase solution. The maximum growth was about the same in each lot, but the growth of the seeds soaked in diastase was very uniform, while that of the water lot varied greatly. The increased vitality of the diastase lot was very noticeable.

He also found that seeds from the same lot that were soaked for fifteen hours in a 3 per cent glucose solution, instead of water, all germinated, thus confirming the view that lack of suitable food was the chief reason why the untreated seed germinated poorly. In this case, there was probably a lack of a starch-inverting enzyme in the seed since equally good results were obtained when either diastase or glucose was supplied.

Among other investigators who have obtained good results working in the same manner may be mentioned A. Thomson,<sup>4</sup> who obtained excellent results on the seeds of barley, oats, corn, peas, white and yellow clover, using 5 per cent pepsin and diastase solutions separately. Others might still be mentioned who have obtained increased germination and vigor of growth of the seedlings by employing these and solutions of other enzymes, but it is not necessary to mention them here.

From the literature at hand, it was found that very few, if any, experiments have been made by supplying ferments or substance which ferments act upon to the growing plant, but the work seems to have been confined chiefly to the germination of seeds and the initial growth of the young seedling. No reference has been found where any work of this kind has been done on tobacco.

In view of the fact that in some recent work<sup>5</sup> the writers have found several enzymes in the growing tobacco and also in the seed, it was thought it might prove of interest to try the effect of supplying dilute solutions of some of the ferments, or of the materials which they act upon, to the young plants to see if they

<sup>3</sup> Wisconsin Agricultural Experiment Station, Research Bulletin No. 22, p. 106.

<sup>4</sup> *Gartenflora*, Berlin, xlv, p. 344, July, 1896.

<sup>5</sup> *Journ. Amer. Chem. Soc.*, xxxv, No 9, September, 1913.

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would promote their growth. It was thought that the seed might be lacking either in a part of the necessary food reserve material or that one or more of the enzymes might not have sufficient activity to promote the desired changes required by the growing plant. A brief outline of the plan of the experiments was as follows:

Before sowing, the seeds were soaked in the solutions of the different substances and after germinating in the soil, the young plants were supplied with fresh dilute solutions of the same until they had reached the transplanting stage in the greenhouse. In the meantime, observations were frequently made as to the uniformity, thickness and growth of the plants in the different boxes; and at the end the plants in each were cut close to the soil and weighed so that more definite results could be obtained in regard to what the experiments had demonstrated.

For the work, a sufficient quantity of virgin bluegrass soil was sterilized by heating at 90°-100°C. for thirty minutes to destroy the weed seed. The soil was then thoroughly mixed and equal quantities of about ten pounds were measured into boxes 12×12×3 inches in size and supplied with good drainage. Two hundred Burley tobacco seeds that had been cleaned were used for each box and each lot was soaked in the different solutions for twenty-four hours, filtered and allowed to dry over night before sowing. The amount of the different substances used in the above solutions, the manufacturer and the box number in the series were as follows:

- No. 1. Burley seed + 2.5 cc. of hydrant water used as a check.
- No. 2. Same as No. 1.
- No. 3. Burley seed + 2.5 cc. of 5 per cent peptone solution (Witte).
- No. 4. Burley seed + 2.5 cc. of 5 per cent diastase of malt solution (Eimer & Amend).
- No. 5. Burley seed + 2.5 cc. of 5 per cent Taka-diastase solution (Parke, Davis & Co.).
- No. 6. Burley seed + 2.5 cc. of a nutritive solution containing 1500 cc. hydrant water + 1 gram  $\text{KNO}_3$  + 0.5 gram  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  + 0.5 gram  $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$  + 0.5 gram  $\text{Ca}_3\text{P}_2\text{O}_8$ .
- No. 7. Burley seed + 2.5 cc. of 5 per cent glucose solution (Eimer & Amend).
- No. 8. Burley seed + 2.5 cc. of 5 per cent trypsin solution (Fairchild Bros. & Foster).
- No. 9. Burley seed + 2.5 cc. of 5 per cent pancreatin solution (Merck, pure).

No. 10. Burley seed + 2.5 cc. of 5 per cent papain solution (Eimer & Amend).

11. Burley seed + 2.5 cc. of 5 per cent casein solution (acc. to Hammarsten).

No. 12. Burley seed that had been treated with  $H_2SO_4$ .

No. 13. Burley seed + 2.5 cc. of a mixture of equal parts of 5 per cent Taka-diastase and 5 per cent pepsin solutions.

No. 14. Burley seed + 2.5 cc. of 5 per cent pepsin solution (Merck, U. S. P., VIII).

No. 15. Burley seed + 2.5 cc. of 5 per cent emulsin solution (Kahlbaum).

No. 16. Burley seed without any previous soaking with water. This was used as a check to compare the unsoaked seed.

No. 6 was carried on so that a comparison might be made of plants that had been supplied with a solution containing all the necessary plant food.

No. 12 was included in order that the plan recommended by Love and Leighty,<sup>6</sup> of acid treatment of some hard coated seeds<sup>7</sup> to insure a quicker and better germination, might be tried. This treatment was as follows: A quantity of seeds were soaked with five or six times their volume of  $H_2SO_4$  (sp. gr. 1.84) for fifteen minutes. Water was then added and decanted quickly, so as to prevent the seed from heating. The seeds were then washed with water until free of acid by litmus paper test and dried.

On January 15, the seeds were mixed with sand and sown in their respective boxes and to each were added 800 cc. of water. After this date, the boxes were watered frequently, adding each time 500 cc. of a fresh 0.01 per cent solution of the substances given above to the respective boxes, except No. 13 to which were added 500 cc. of a solution containing 0.005 per cent of each ferment. Of course No. 6 was watered only with the nutritive solution and Nos. 1, 2, 12 and 16 with water. To each box, however, the same volume of 500 cc. was added every time. The nutritive solution was not used constantly but alternated with water. Hydrant water was used throughout the experiments.

From the beginning Nos. 14, 13 and 11 gave the best growth and Nos. 14 and 11 maintained this lead until the plants were weighed. Nos. 10, 15, 9 and 7 also showed up well from the first. Nos. 6 and 3 made good gains in the last few weeks of the

<sup>6</sup> Bulletin 312, Cornell Agric. Exp. Station, p. 335.

<sup>7</sup> Tobacco seeds were not used in Love and Leighty's experiments.

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experiment. After January 25, the strength of each solution except No. 6, was reduced to one-half of that formerly used because it was thought that this would lessen the chances of fungi developing in the boxes.

The boxes were watered on an average two and one-half to three times a week using the same amount of water or solution during the first part of the experiment. Afterwards it was found that the boxes which were making the better growth appeared to retain the water longer than the others. The plan was then followed of adding extra water to the dry boxes so as to keep all at about the same degree of wetness. Consequently the checks and those boxes which gave the poorest growth had more water added.

It is interesting to note that the boxes which gave the better growth of plants were the ones which seemed to retain the moisture longer and this was true from the beginning. This was very noticeable during the first part of the experiment on comparing these boxes with the checks. It would be expected that as the result of the larger growth of the plants and therefore of their larger water requirement that these boxes would have dried out quicker than those containing the smaller plants, but the reverse seemed to take place. There might be a possible reason for this when the plants attained sufficient size to shade the soil and enable it to hold the water, but even then it would more than likely be counterbalanced by the increased size of the plants. Certainly this explanation would not hold true when all the plants were very small. While the foregoing from frequent observations appeared to be true, further work is required to prove this interesting point.

About the first of March, approximately the same number of plants in each were transplanted and arranged in their respective boxes so that they would be more evenly distributed in the soil.

On March 15, the plants in several of the boxes were of sufficient size to be transplanted. At this time, No. 14 had the largest plants. These appeared to be more uniform in size and thicker in the box. Next in order were Nos. 10, 11 and 15. Then came Nos. 3, 6, 7, 8 and 13 with the plants as evenly distributed and of uniform size as in the boxes mentioned above but somewhat smaller. Next in order was No. 16, and finally Nos. 1, 2, 4 and

5 had about the same appearance. In Nos. 1, 2, 4, 5 and 16 the plants did not come up with an even stand and were as a rule much smaller than the rest. No. 16, however, made a very good growth towards the end and gained on some of the other boxes. This may have been due to the fact that the plants in this box, not being as thick as those in some of the others, had more room in which to grow.

On March 21, the plants were cut off close to the ground and those in each box weighed separately in order to see what differences would be shown in the individual weights. The results were as follows:

No.	Grams	No.	Grams	No.	Gram
1 (check).....	55	6.....	128	11.....	139
2 (check).....	92	7.....	116	12.....	—*
3.....	113	8.....	127	13.....	116
4.....	91	9.....	96	14.....	174
5.....	89	10.....	126	15.....	138
				16 (check).....	120

\* Not weighed: only two seeds germinated.

The above weights of the plants in the different boxes are in fairly close agreement with the conclusions reached as to their general appearance before cutting. The differences between the checks Nos. 1 and 2 are larger than desirable but differences like these will occur in work of this kind and are difficult to explain. The differences between Nos. 1, 2 and 16, all check boxes, are again large and in favor of the unsoaked seed (No. 16), which is contrary to what would be expected. One explanation, although hardly a plausible one, as to why this may be true, is that soaking the seed before planting may have extracted some soluble reserve material from it which the plant needs for its growth. The differences between Nos. 1 and 2, according to this supposition, may have been caused by the water extracting more of this material from one lot of seeds than from the other. The growth of the plants in Nos. 1, 2 and 16 was in harmony with this theory since No. 16 maintained the lead over the others almost from the beginning.

The proper checks on this series are Nos. 1 and 2 and, taking the better or No. 2, the differences between this and some of the best, for example, Nos. 11, 14 and 15 are very large. In only

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three, Nos. 4, 5 and 9 were the results about the same as this check while the remainder show consistent gains over it.

The above results may be due to some fertilizer constituent that was added in the solutions. On the other hand, they may be due to some deficient substance or ferment that was supplied to the plants.

As it is almost impossible to obtain the active ferment free from protein material, it was thought that the total nitrogen in the substances used would probably explain the differences obtained in the growth of the plants. Accordingly, nitrogen determinations were made on all the materials by the modified Kjeldahl method so that any nitrate nitrogen, if present, would be included.

For convenience, the materials used, the weight of the green plants, the increase over check No. 2, the amount of substance supplied and of nitrogen in the same, are given in Table I.

TABLE I.

SUBSTANCE	WEIGHT OF PLANTS WHEN CUT		GAIN IN WEIGHT OVER CHECK NO. 2	WEIGHT OF SUBSTANCE ADDED		NITROGEN IN SUBSTANCE ADDED	TOTAL WEIGHT OF NITROGEN ADDED
	grams	per cent		grams		per cent	gram
No. 1. Check.....	55						
No. 2. Check.....	92						
No. 3. Peptone.....	113	22.8	0.550			15.880	0.0673
No. 4. Malt diastase.....	91	-1.1*	0.550			6.040	0.0332
No. 5. Taka-diastase.....	89	-3.3*	0.550			1.400	0.0077
No. 6. Nutritive solution.....	128	39.2	5.000 of KNO <sub>3</sub>			13.860	0.6930
No. 7. Glucose.....	116	26.1	0.550			0.640	0.0035
No. 8. Trypsin.....	127	38.0	0.550			12.260	0.0674
No. 9. Pancreatin.....	96	4.3	0.550			12.580	0.0692
No. 10. Papain.....	126	37.0	0.550			1.560	0.0086
No. 11. Casein.....	139	51.1	0.550			14.640	0.0805
No. 12. Seed treated with H <sub>2</sub> SO <sub>4</sub> .....							
No. 13. { Taka-diastase } { Pepsin }	116	26.1	{ 0.275 0.275 }			1.400	0.0039 5.140
No. 14. Pepsin.....	174	89.1	0.550			5.140	0.283
No. 15. Emulsin.....	138	50.0	0.550			10.500	0.0578
No. 16. Check.....	120						

\* Loss.

## CONCLUSIONS.

After a period of two months, it appears from the above table that marked differences can be observed in regard to the growth made by some of the plants: For instance, pepsin, casein and emulsin made larger and more uniform plants than the rest. It is interesting to note the good results obtained from the use of emulsin, and in this connection it might be mentioned that good tests were obtained for this enzyme in the Burley seed and growing plant in some previous work.

In every case, with the exception of two, all the boxes were better than the two checks, Nos. 1 and 2, which properly belong to the series and one-half of the boxes were better than the third check, No. 16. As a rule, the proteolytic ferments and the protein substances gave the best growth. The results obtained with trypsin and pepsin are contrary to what would be expected since it is doubtful if the latter is present in plants. Pepsin is regarded as an animal ferment and works best in an acid medium. No positive results were obtained with the diastase ferments.

The results in all cases cannot be explained from the total nitrogen added, since pepsin, which gave the best growth, has only 5.14 per cent of nitrogen, whereas some of the others which did not have so great an effect, as pancreatin, peptone and trypsin, contain respectively 12.58, 15.88 and 12.26 per cent of nitrogen. Again, on comparing pepsin which gave 89.1 per cent increase of growth with malt diastase which showed a decrease of 1.1 per cent, we find that the total nitrogen in the two samples is 5.14 per cent and 6.04 per cent respectively with the advantage in favor of the latter.

To No. 6 was supplied in the nutritive solution a total of 5 grams  $\text{KNO}_3$ ; 2.5 grams  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ; 2.5 grams  $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$  and 2.5 grams  $\text{Ca}_3\text{P}_2\text{O}_8$ . This box was certainly supplied with abundant plant food, and while it made a steady growth throughout the experiment, nevertheless pepsin, emulsin and casein made better growths with much smaller amounts of nitrogen supplied. Papain, trypsin and the nutritive solution gave about the same results yet the nitrogen added to each was 0.0086, 0.0674 and 0.6930 gram respectively.

The question of the availability of the nitrogen in the various

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substances might explain some of the differences. In this case, the nitrogen supplied in the form of potassium nitrate in the nutritive solution should be readily available. Again, nitrogen in peptone would likely be regarded as more available than in casein yet casein gave a much better growth with less nitrogen added.

Lack of an opportunity prevented any further work from being done on the samples to find if any other fertilizer constituent had been added in appreciable amounts. Granting that some potassium and phosphorus were added in these substances, on the other hand considerably larger amounts of these elements in available form were added in the nutritive solution.

The acid treatment of tobacco seed according to the method used was not satisfactory since only two germinated. The acid was either too strong or the time allowed for soaking the seed too long and this permitted the outer coats to be broken up and partly dissolved, thus allowing the strong acid to come in contact with and kill the embryo.

The substances used in this work were recently obtained from the manufacturer but no previous tests were made on the different ferments to determine their degree of activity.

The plants did not grow as rapidly in the greenhouse as they generally do in the plant bed. When they were cut, the plants in some of the best boxes mentioned above which had reached the transplanting stage were two to three weeks in advance of checks Nos. 1 and 2.

While these experiments are only preliminary, nevertheless some of the results are interesting inasmuch as they indicate benefits that might be acquired in practice. It is intended to continue this work when an opportunity affords and compare these and other materials in which active enzymes are present and the same substances in which they have been destroyed by heat.

### PART II.

Many experiments have been made by different investigators on the effect of adding certain substances known as catalytic fertilizers to plants to promote their growth. These substances are generally spoken of under the above title inasmuch as growth



is promoted by their presence and since, as is assumed, they do not necessarily enter into the chemical composition of the plant, they cannot be regarded as plant food materials. They are also referred to as plant stimulants.

Among the substances that have been commonly used are salts of certain metals, such as iron, aluminium, manganese, etc., and while it is only necessary to refer briefly to the literature in regard to the work which has been done along this line, the results of numerous experiments might be mentioned where marked differences in the growth of different plants were obtained on the addition of certain substances to the soil. This is especially true in regard to the use of manganese salts.

The work is in the experimental stage but it opens up an interesting field of investigation in view of the fact that these metals are commonly found in plants and in much larger amounts in the soil. No importance has ever been attached to them other than the fact that one or two are included in the list of the essential elements required by plants, but since they are found in considerable amounts in the soil, it has been assumed that it is not necessary to add them in practice.

Since Euler<sup>\*</sup> mentions the fact that certain substances (for example iron or manganese salts) accelerate the action of some enzymes and as several ferments have been found in the tobacco, it occurred to one of us (Shedd) that it might prove of interest to try the effect of some of these substances on the growth of Burley tobacco plants. It is possible that the good effects of the catalytic fertilizers may be partly due to an acceleration of the enzyme activity in the plant.

For these experiments, iron and manganese salts of citric, malic and oxalic acids were used because it was thought that since these acids have been found in tobacco, their salts would not have a tendency to retard the growth of the plant. Besides the above substances, weak solutions of hydrocyanic acid and potassium cyanide were used in the series in two boxes to determine their effect on the plant. As emulsin has been found in tobacco, it was thought that the addition of these substances might prove interesting since it is generally recognized that small amounts of

<sup>\*</sup> Euler-Pope: *General Chemistry of the Enzymes*, 1912, pp. 108-10.

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strong poisons sometimes act as powerful stimulants. Amygdalin was also tried alone in this series. As a continuation of the preceding series, mixtures of the substrate and the ferments were also included and finally another trial was made of the sulphuric acid treatment of the seed, except in this case the seed was soaked for only three minutes instead of fifteen as before.

The experiments were carried out in the same manner as those in the preceding series, except the seed was not soaked before planting. The same kind of soil prepared as previously described was used. Two hundred seeds, from the same lot used before, were sown in each box.

The materials used in the series were as follows:

- No. 1. Check.
- No. 2. Casein + trypsin + pepsin.
- No. 3. Pancreatin + peptone + glucose.
- No. 4. Sulphuric acid treatment of seeds.
- No. 5. Iron and manganese carbonate (Merck).
- No. 6. Iron and manganese peptonate (Eimer & Amend).
- No. 7. Manganese citrate (Merck).
- No. 8. Iron malate (Eimer & Amend).
- No. 9. Iron citrate, U. S. P. (Eimer & Amend).
- No. 10. Iron (ous) oxalate, pure (Eimer & Amend).
- No. 11. Potassium cyanide (Eimer & Amend).
- No. 12. Hydrocyanic acid (Eimer & Amend).
- No. 13. Iron and manganese lactate (Eimer & Amend).
- No. 14. Amygdalin.

The seeds were planted February 5, 1913, and the boxes were watered each time with 500 cc. of the following solutions made with hydrant water from the above substances. The substances given above which were used in the preceding series, were taken from the same samples used before.

- No. 1. 500 cc. hydrant water.
- No. 2. 500 cc. of a solution containing 0.0025 per cent of each substance.
- No. 3. 500 cc. of a solution containing 0.0025 per cent of each substance.
- No. 4. 500 cc. hydrant water.
- No. 5. 500 cc. of a 0.01 per cent solution.
- No. 6. 500 cc. of a 0.01 per cent solution.
- No. 7. 500 cc. of a 0.01 per cent solution.
- No. 8. 500 cc. of a 0.01 per cent solution.
- No. 9. 500 cc. of a 0.01 per cent solution.
- No. 10. 500 cc. of a 0.01 per cent solution.

No. 11. 500 cc. of a HCN solution containing 1.03 parts HCN per million.

No. 12. 500 cc. of a KCN solution containing 2.47 parts KCN, i.e., 1.03 parts HCN per million.

No. 13. 500 cc. of a 0.01 per cent solution.

No. 14. 500 cc. of a 0.005 per cent solution.

In Nos. 2, 3, 11, 12 and 14 the solutions were made fresh each time, while in the others stock solutions of 2500 cc. were made as needed.

From the beginning, Nos. 3, 10, 11 and 12 were much better in appearance than the others and No. 10 had considerably more seeds to germinate in less time than the rest. Only a few seeds, not over eight or ten, germinated in No. 4 and this box was discarded.

On February 28, Nos. 3, 9, 10, 11, 12, 13 and 14 were better than the check but unfortunately some mice destroyed most of the large plants in these boxes, especially No. 10 in which the tops were gnawed close to the ground.

On March 6, the strengths of the HCN and KCN solutions were increased to 2.06 parts HCN per million and from this time about every three days, the strength was increased about 2 parts per million each time until on March 18, the solutions contained 10.3 parts HCN per million and these were used until the end of the experiments.

On March 24, the plants were transplanted and arranged in the boxes so as to evenly distribute them in the soil. At this time, in general appearance, No. 11 was in the lead; next in order were Nos. 6 and 12; then Nos. 3 and 13 were about the same; Nos. 2, 5, 7 and 10 were about like the check and Nos. 8, 9 and 14 appeared to be behind it.

On April 11, the plants in the best boxes were of sufficient size for transplanting. At this time they ranked in general appearance as follows: Nos. 6 and 11 were the best. The plants in No. 6 were more uniform and probably were a little better than No. 11, although the latter had some larger plants. Next in appearance were Nos. 3, 12 and 13. The plants in these boxes were not so very uniform especially in No. 12 which had some large plants and some very small ones. Nos. 6 and 13 had made good growth during the preceding few days. Next in order were

## 452 Effect of Ferments on Growth of Tobacco

Nos. 7 and 10 which were fairly uniform and looked slightly better than No. 1. Nos. 2 and 8 appeared about the same as the check. There were fewer plants in No. 8 but they were larger than those in No. 1. In No. 2 the plants were uniform while in the check they were not, which made the comparison very difficult. Nos. 5, 9 and 14 did not show up as well as the check.

On this date, the plants were cut close to the ground and weighed. For purposes of comparison, the weights of the green plants, the gain or loss in weight compared with the check, and the amounts of the different substances and nitrogen added in the same are given in Table II.

TABLE II.

NUMBER AND SUBSTANCE	WEIGHT OF PLANTS WHEN CUT	GAIN OR LOSS COMPARED WITH NO. 1	WEIGHT OF SUBSTANCE ADDED		NITROGEN IN SUBSTANCE ADDED	
			grams	per cent	grams	per cent
1. Check.....	95					
2. { Casein.....	92	-3.2	0.2625	14.64	0.0384	
Trypsin.....			0.2625	12.26	0.0322	
Pepsin.....			0.2625	5.14	0.0135	
Pancreatin.....	114	+20.0	0.2625	12.58	0.0330	
3. { Peptone.....			0.2625	15.88	0.0417	
Glucose.....			0.2625	0.64	0.0017	
4. Seeds treated with H <sub>2</sub> SO <sub>4</sub> .....						
5. Iron and manganese carbonate.....	69	-27.4	1.0500			
6. Iron and manganese peptonate.....	142	+49.5	1.0500	10.32	0.1084	
7. Manganese citrate.....	91	-4.2	1.0500			
8. Iron malate.....	75	-21.1	1.0500			
9. Iron citrate.....	60	-36.8	1.0500			
10. Ferrous oxalate.....	82	-13.7	1.0500			
11. KCN.....	126	+32.6	0.1235	21.52*	0.0265	
12. HCN.....	108	+13.7	0.0512	51.85*	0.0265	
13. Iron and manganese lactate.....	105	+10.5	1.0500			
14. Amygdalin.....	62	-34.7	0.5250	3.06*	0.0161	

\* Calculated.

### CONCLUSIONS.

It is of interest to note in this series, which was carried on in the same manner and for the same time as the preceding one, that pepsin in combination with casein and trypsin failed to show

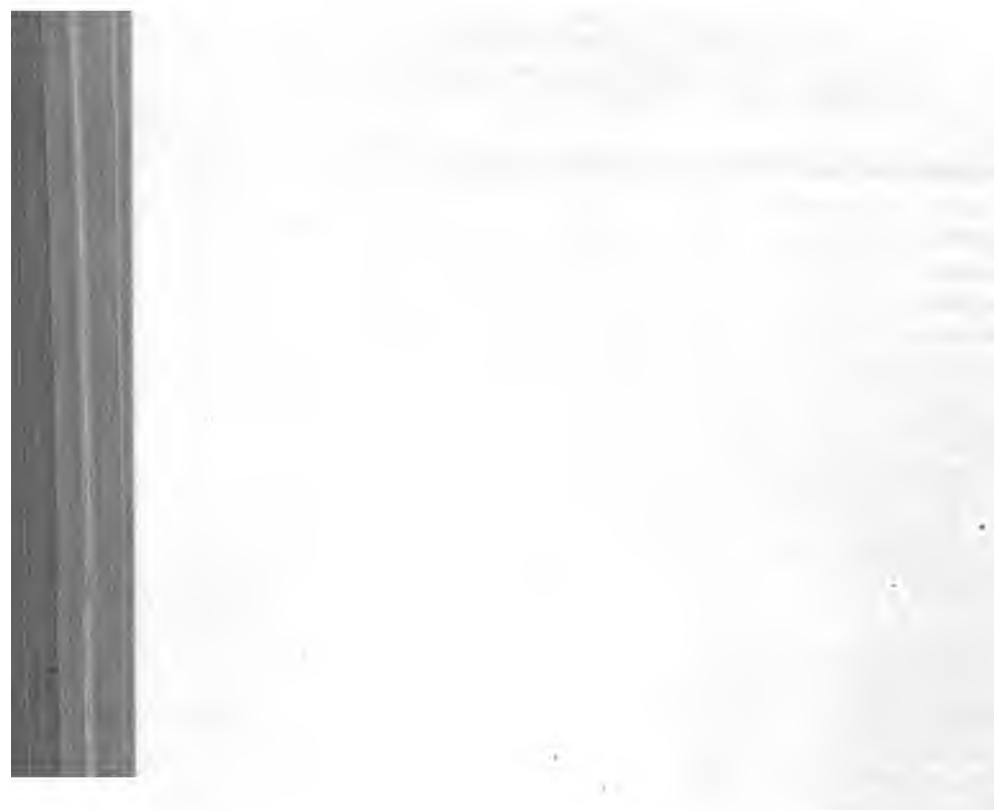
any increase of growth, whereas alone each substance before gave a decided gain. In No. 3, the combination of the three substances shows a material gain and each also gave an increase in the preceding series.

Of the other substances, iron and manganese peptonate, potassium cyanide, hydrocyanic acid and iron and manganese lactate gave positive results and in the order named. The remainder gave negative results and in some cases the losses were very large.

The results cannot be explained as due to the nitrogen added since some of the good boxes had much smaller amounts supplied to them than those in which the losses were very large. This becomes more apparent on comparing this and the former series in so far as pepsin is concerned.

The good results obtained with the potassium cyanide and hydrocyanic acid are worthy of further study and it would be interesting to find just what amounts of these substances the plant can stand in order to obtain its best growth and the effect, if any, they may have on its composition and quality.

In conclusion, the writers desire to express their appreciation to Dr. Joseph H. Kastle for suggesting a part of this work and for valuable advice given during its progress.



## STUDIES ON THE THEORY OF DIABETES.

### II. GLYCID AND ACETOLE IN THE NORMAL AND PHLORHIZINIZED ANIMAL.

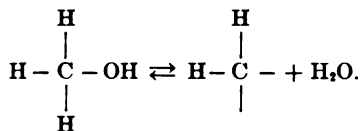
By J. R. GREER, E. J. WITZEMANN AND R. T. WOODYATT.

(From the Otho S. A. Sprague Memorial Institute Laboratory of Clinical Research, Rush Medical College, Chicago.)

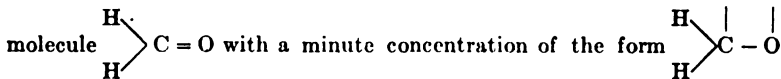
(Received for publication, October 27, 1913.)

Many organic chemical reactions are most simply explained by the theory that organic substances are in part dissociated to give residues which are in a state of dynamic chemical equilibrium with the undissociated molecules. Indeed for the explanation of an equilibrium such as that seen when sugars are dissolved in weak alkali some such theory is absolutely indispensable.

This conception has been developed elaborately by Nef according to whose view methyl alcohol, for instance, consists of a great preponderance of undissociated alcohol molecules in dynamic chemical equilibrium with a very minute quantity of methylene and water

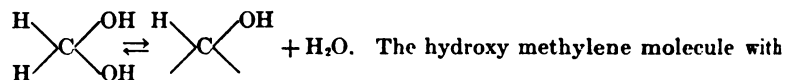


The addition of an alkali like KOH leads to the formation of a salt  $\text{CH}_2\text{OK}$  (potassium alcoholate) the dissociation of which into KOH and methylene, is greater than that of alcohol into HOH and methylene, so that the effect of alkali is to raise the reactivity of methyl alcohol by increasing the concentration of methylene. Similarly, formaldehyde is held to consist under ordinary circumstances chiefly of the



which, with H and OH, gives the hydrate  $\begin{array}{c} \text{H} \\ \diagup \\ \text{C} \\ \diagdown \\ \text{H} \end{array} \begin{array}{l} \text{O}-\text{H} \\ \text{O}-\text{H} \end{array}$  and this, in a

manner analogous to that shown by methyl alcohol, dissociates with the loss of water into hydroxy methylene and water, thus:



an excess of available H becomes methyl alcohol; with an excess of OH (or O), formic acid; with H and OH in equal quantity it regenerates the aldehyde, etc. In short, *formaldehyde first dissociates and the fate of the dissociated particles depends upon the character of the reaction mixture in which they find themselves.* Alkali, heat, light, certain enzymes, etc., serve to increase dissociation.<sup>1</sup>

Multiplication of experiments makes it possible in many cases to recognize with considerable definiteness the actual configuration of the unsaturated residues concerned. Yet in the interpretation of metabolic phenomena this system of chemical thought has found little application. Ringer<sup>2</sup> holds for incorrect Neubauer's conception<sup>3</sup> of oxidative deaminization of alanine and his belief that when alanine is broken down in the body, pyruvic acid is the main first "*fassbares*" product. This dissent is based on the fact that alanine in a fully phlorhizinized dog goes over almost quantitatively into sugar while pyruvic acid does so in some cases only to a limited extent if at all. But pyruvic acid, according to Embden, will yield lactic acid in a perfused liver and lactic acid will go over quantitatively into sugar in a phlorhizinized dog. The point which looms clearly out of these experiments would seem to us to be that alanine is first *dissociated* and that the dissociated residue may make lactic acid under conditions in which there is an equal concentration of OH and H, *i.e.*, when free O is deficient (Embden's perfusions), or form pyruvic acid where O is in excess and the whole bodily equilibrium is not upset in the direction of glucose (Neubauer's oxidative deaminization) or form glucose (via methyl glyoxal or lactic acid?) when the rapid withdrawal of glucose from the body upsets the entire chemical equilibrium of the metabolites in this particular direction (phlorhizin diabetes).

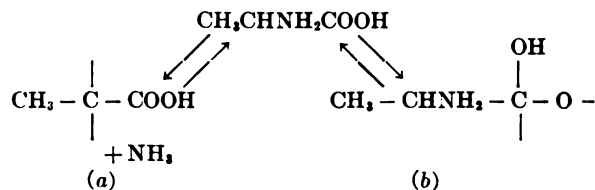
<sup>1</sup> Cf. *Liebig's Annalen*, cccxxxv, p. 191 *et seq.*

<sup>2</sup> This *Journal*, xv, pp. 145-52, 1913.

<sup>3</sup> *Deutsch. Arch. f. klin. Med.*, xcv, p. 211.



One might assume, with a mass of chemical data to support the view, that alanine is dissociated in at least two ways, as follows:



Molecule *a* with H and OH gives lactic acid;<sup>4</sup> with 2OH or O, pyruvic acid. Molecule *b* represents the phase which with an excess of H permits the reduction of the carboxyl group by addition of 2 H's and subsequent loss of H<sub>2</sub>O, etc.

Again Embden assumes that because the trioses (*e.g.*, glyceric aldehyde) are capable of forming lactic acid by the action of surviving muscle, leucocytes, blood plasma, etc., that "Milchsäure ein auf dem Hauptwege des Traubenzuckerabbaus gelegenes Product sei."<sup>5</sup> But all of Embden's experiments were carried out under what may be termed *asphyxial* conditions, and prove only that *when there is a lack of free oxygen* lactic acid is a chief product of the breakdown of the glucose. An *asphyxiated* alkaline glucose solution *in vitro* also forms lactic acid, but the latter is certainly no intermediate in the *fully oxygenized* alkaline glucose solution since no lactic acid appears in a fully aerated alkaline sugar solution, whereas preformed lactic acid is not destroyed when added to such a mixture. This experiment, performed by Meisenheimer and by Nef, we have amply confirmed. Glyceric aldehyde is rather first dissociated, and whether the residue burns or goes into lactic acid via methyl glyoxal will depend upon the conditions of the experiment.

The following experiments with acetole and glycid were performed for the purpose of studying their behavior in the body apart from any theory but with special reference to the applicability of the above-mentioned views.

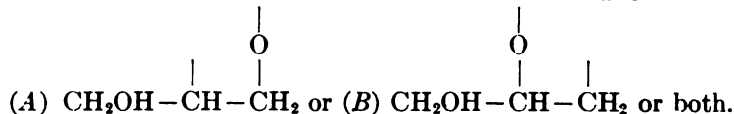
<sup>4</sup> The simultaneous occurrence of types *a* and *b* with intramolecular rearrangement can yield methyl glyoxal—which by a benzilic acid rearrangement may give lactic acid.

<sup>5</sup> *Biochem. Zeitschr.*, xlv, p. 108, 1912.

*Glycid.*

$$\begin{array}{c} \text{O} \\ \diagup \quad \diagdown \\ \text{CH}_2\text{OH}-\text{CH}-\text{CH}_2 \end{array}$$

Glycid,  $\text{CH}_2\text{OH}-\text{CH}-\text{CH}_2$ , is an internal ether, representing a class of substances which, so far as we know, has not received attention from the biological standpoint, owing perhaps to the difficulties attending their preparation. Since the purely chemical behavior of these substances resembles that of the ordinary ethers, a parallelism might be anticipated in the body. Glycid does not reduce Fehling's or other sugar test solutions<sup>6</sup> although it is generally stated to the contrary in the literature, and when boiled with two parts of water for eight hours, passes over into glycerol. This hydration implies a preliminary rupture of the ring with the intermediate existence of the unsaturated residue



Consequently if the ring in glycid were opened in the body, the biological effect of these residues might be determined. When glycid (or glycidic acid) is treated with halogen acids, the halogen is added chiefly to an end C atom, giving  $\text{CH}_2\text{OH}-\text{CHOH}-\text{CH}_2\text{Cl}$  (or  $\text{COOH}-\text{CHOH}-\text{CH}_2\text{Cl}$ ) which is interpreted most simply by assuming that under these conditions the dissociation which yields the molecule B is the predominating one. Furthermore when glycid is heated ( $450^\circ$ ) it forms acetole  $\text{CH}_2\text{OH}-\text{CO}-\text{CH}_3$  which is also ascribable to the

$$\begin{array}{c} \text{O} \qquad \qquad \text{O} \\ | \qquad \qquad | \\ \text{CH}_2\text{OH}-\text{CH}-\text{CH}_2 \end{array}$$

B residue, thus:  $\text{CH}_2\text{OH}-\text{CH}-\text{CH}_2 \rightarrow \text{CH}_2\text{OH}-\text{C}(=\text{O})-\text{CH}_3$  (Nef). If in the body the ring remained intact, the biological effects should resemble those of ethers in general. As a matter of fact the biological behavior of glycid is in harmony with the idea that the ring is but little attacked in the body.

*Material.* The glycid used was prepared by treating  $\alpha$ -chlorhydrin with alcoholic KOH in the cold, and subsequent fractionation. It was a clear glycerol-like fluid of sp. gr. 1.10 (Westphal); b. p.,  $62^\circ\text{C}$ . at 15 mm. pressure, and corresponding otherwise

<sup>6</sup> Nef: *Liebig's Annalen*, cccxxv, p. 232.

entirely with the product described by Nef.<sup>7</sup> It had a slight aromatic odor and mild taste.

*Animal experiments with glycid.* Glycid was given subcutaneously to guinea pigs and rabbits. When 1 gram of glycid was given to a pig of 519 grams body weight (1.9 grams per kilo) no effects were at first apparent. After fifteen minutes the animal ceased feeding and became sluggish. In forty-five minutes it was very dull and could scarcely be aroused. There was slight twitching of the legs at this time, although it lay soporose in the natural position. In two hours it was dead without having changed its position except for the settling as the tone left the muscles. Post mortem examination showed an engorged right heart and lungs, a few subserous ecchymoses, the liver pale, with some fatty infiltration and parenchymatous degeneration, kidneys congested. Otherwise no change of note.

Dosages of 1 gram per kilo of weight were usually lethal. With 0.4 gram per kilo the animals became dull, but later recovered. In some fatal cases the parenchymatous changes in the liver and kidneys were more definite and the tendency to small hemorrhages greater so that the picture resembled chloroform poisoning. Death appeared to result from failure of respiration. Owing to the toxicity of glycid, no satisfactory results with phlorhizinized dogs were obtained.

The molecules *A* or *B* if formed in the body by opening of the ring, would be closely related to glycerol or acetole, neither of which is so highly toxic as glycid. These poisonous effects are accordingly ascribed to the preservation of the ring, *i.e.*, to the maintenance of the molecular form of an internal ether whose biological effects resemble those of other ethers.

#### *Acetole.*

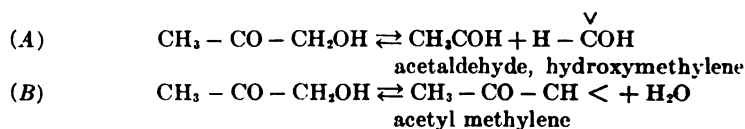
Acetole (hydroxy acetone)  $\text{CH}_2\text{OH}-\text{CO}-\text{CH}_3$  stands between acetone on one side and the ketotriose, dihydroxy acetone, on the other. According to Emmerling and Loges,<sup>8</sup> it is formed when hexoses are fused with caustic alkali and it has accordingly been held by certain writers to be an intermediate in the break-

<sup>7</sup> *Loc. cit.*, p. 232.

<sup>8</sup> *Ber. d. deutsch. chem. Gesellschaft.*, xvi, p. 837.

down of sugars into lactic acid under the influence of alkali, and a similar rôle has been suggested for it in the body. It is the alcohol corresponding to pyruvic acid and methyl glyoxal (pyruvic aldehyde), concerning the former of which discussion is now in progress, and is isomeric with lactic aldehyde  $\text{CH}_3-\text{CHOH}-\text{COH}$

and glycid  $\text{CH}_2\text{OH}-\text{CH}-\text{CH}_2$ . Acetole has not heretofore been directly tested in biological experiments. Its many known reactions are conveniently referable to two types of dissociation.<sup>9</sup>



The occurrence of the former type, *A*, is supported by the formation of acetaldehyde and metaformaldehyde when acetole is passed through heated tubes (at 450°) and by the formation of acetic and formic acids when acetole is oxidized with mercuric or silver oxide or with chromic and dilute sulphuric acids. The type *B* is suggested by Nef as the basis for the condensation of acetole and of analogous substances with strong alkali. Moreover when acetole is treated with copper acetate at 130° the copper salt is reduced and cuprous oxide is formed, the acetole passing over mainly into lactic acid. This lactic acid formation Nef ascribes to the formation from molecule *B* of acetyl formaldehyde (methyl glyoxal),  $\text{CH}_3\text{CO}-\text{CH} < + \text{O} = \text{CH}_3-\text{CO}-\text{COH}$ , which then by a benzilic acid rearrangement becomes lactic acid  $\text{CH}_3-\text{CHOH}-\text{COOH}$ . But when acetole is treated with alkali alone, no lactic acid, or only traces, occur. Consequently acetole cannot be an intermediate step in the formation of lactic acid by the action of alkali alone on hexoses. This lactic acid comes rather from methyl glyoxal by the process just mentioned—the latter substance being formed when alkali acts on hexoses *in the absence of oxygen*.

It follows from the foregoing that if a fundamental parallelism exists between the behavior of sugars in alkaline solution and in the body, as we have found it practical to assume, then acetole would not be an intermediate in the catabolism of glucose. Secondly, if the behavior of acetole itself in the body is analogous to

<sup>9</sup> *Liebig's Annalen*, cccxxxv, p. 250.

that observed in the presence of metallic oxides in weakly alkaline solution, its fate in the body should be chiefly that of acetaldehyde and hydroxy-methylene in accordance with the scheme *A*.

Now if acetole were convertible into lactic acid in the body by any process at all, the lactic acid so formed would be capable of yielding glucose in a fully phlorhizinized dog. This is equivalent to saying that if acetole by its dissociation could yield *B* particles which were convertible into lactic acid, acetole would be convertible into glucose. A failure on the part of acetole to form sugar would also speak against the dissociation *B* since this dissociation would yield methyl glyoxal (pyruvic aldehyde) which has been shown by Dakin and Dudley to be a sugar former.

The experiments with acetole warrant the conclusion that this substance is not a sugar former when given either by mouth or subcutaneously to phlorhizinized dogs, and not a normal intermediate between  $C_6H_{12}O_6$  and  $C_3H_6O_3$  (because all  $C_3H_6O_3$  compounds are sugar formers regardless of their configuration; dihydroxy acetone [Mostkowskii], glyceric aldehyde [Woodyatt], lactic acid [Lusk], hydracrylic acid). The results are most simply explained by assuming that acetole dissociates in the body in accordance with the scheme *A*, viz.,  $CH_3CO-CH_2OH \rightleftharpoons CH_3CO + CHO$ , correspond-



ing to that seen in the presence of weakly alkaline metallic oxides *in vitro*.

In the experiments in which acetole was first given to phlorhizinized dogs it was noticed that following its administration the urine gave the characteristic Gerhardt reaction with ferric chloride in dilutions twice as high as before. A definite increase was also noted in the difference between the polariscopic and titration estimations of sugar. This suggested an increase of the acetone bodies and in another experiment (II)—in which these were followed—the suspicion appears to have been confirmed, although the rising acidosis may have been a mere incident in the diabetes. An increase of acetone bodies may be attributed to aldole formed from acetaldehyde.<sup>10</sup>

*Material.* Acetole was prepared from bromacetone and sodium formiate in accordance with the method of Nef.<sup>11</sup> The product

<sup>10</sup> Cf. A. Magnus-Levy: *Arch. f. exp. Path.*, xlv, p. 433, 1901.

<sup>11</sup> *Liebig's Annalen*, cccxxxv, p. 260, *et seq.*

has a sweet taste and was colorless when freshly made but developed a straw color on standing. That used for the experiments was freshly distilled, and the portion used which passed over between 53° and 56° at 20 mm. pressure. For its detection in the urine we made use of the following properties: (a) volatility; (b) power to reduce metallic oxides in alkaline solution in the cold; (c) formation of a hydrazone (with phenylhydrazine); (d) lack of optical activity; (e) failure to give a color with Schiff's reagent for aldehydes.

*Methods.* Glucose in the urine was determined by polariscope and by the method of Bang and Bohmannsson, nitrogen by Kjeldahl; acetone and acetoacetic acid, Messinger;  $\beta$ -hydroxybutyric acid, Shaffer's method applied to the ether extract.

Phlorhizin was given as described in a previous paper.

*Preliminary experiments with acetole.* Healthy guinea pigs, rabbits and dogs received as high as 2 grams per kilo of body weight without fatalities. Nevertheless even 1 gram per kilo often produced symptoms. Following the ingestion or subcutaneous administration of acetole the urine becomes dark and contains then a little albumin and gives the characteristic absorption spectrum of haemoglobin. No reducing substance was found in the urine of a 10 kgm. dog after the administration of 20 grams of acetole by mouth, nor any other product of its decomposition. Acetole causes definite injury to the kidneys and this feature is also evident in the two following experiments.

**EXPERIMENT I.** Fully phlorhizinized fox terrier. Acetole 10 grams in 20 cc. of water made faintly alkaline with  $\text{Na}_2\text{CO}_3$  administered subcutaneously at beginning of fourth 6-hour period.

PERIOD	DEXTROSE			N	D:N*	REMARKS
	Polariscope	Titration	Difference			
I	7.56	8.44	0.88	1.50	5.62	
II	6.20	7.20	1.00	1.95	3.77	
III	5.62	7.20	1.60	2.14	3.36	
IV	5.60	8.27 (7.27)	2.67 (1.67)	2.03	4.07	Urine dark, smoky with trace albumin.
V	4.31	5.31	1.00	1.41	3.76	Urine still darker, contains haemoglobin.

\* D:N based on titration figures for dextrose.

The urine of period IV reduces Haines' solution in the cold. The cold reducing power measured by Bang's solution (one-half hour of contact) corresponds to 1 gram of dextrose. The ability of the urine to reduce in the cold is removed by boiling. The distillate contains a substance which reduces Haines' solution and silvers the walls of tubes containing ammoniacal silver oxide at room temperature. There is no reaction for aldehyde in this distillate with Schiff's reagent. With phenylhydrazine and paranitrophenylhydrazine, hydrazones were obtained from the distillate corresponding in appearance with those prepared for purpose of comparison from pure acetole. The extra reducing substance is therefore attributed to acetole.

EXPERIMENT II. Fully phlorhizinized fox terrier, weight 13 kg., which received 20 grams acetole dissolved in water, by mouth, at beginning of fifth 6-hour period.

PERIOD	DEXTROSE			N	D:N*	ACETONE AND ACETOACETIC ACID	$\beta$ -HYDROXYBUTYRIC
	Polariscope	Titration	Difference				
I	11.78	13.75	1.97	1.86	7.39		
II	9.55	11.88	2.33	1.86	6.39	0.053	0.226
III	8.03	10.30	2.30	2.22	4.65	0.079	0.422
IV	6.00	8.55	2.55	2.28	3.75	0.132	0.951
V	4.65	7.50	2.85	1.96	3.83	0.275	0.997

\* D:N based on titration figures for dextrose.

During the sixth period in this experiment the dog died. The urine after the administration of the acetole was smoky as usual and the distillate contained reducing substance which answered the reaction for acetole as in the previous experiment. Aldehyde was not demonstrable in the urine.

#### SUMMARY.

Glycid and acetole have been prepared in pure form and administered to healthy animals and to fully phlorhizinized dogs.

Glycid is toxic. Doses of 0.3 to 0.4 gram per kilo of body weight cause narcosis, accompanied at times by muscular twitching. Larger doses cause death. The effects are ascribed to the ring, which is opened in the body with difficulty.

Acetole is relatively non-toxic. Doses of 2 grams per kilo of body weight do not kill, but even moderate doses cause haematuria and haemoglobinuria. When given to phlorhizinized dogs either subcutaneously or by mouth, acetole causes no output of extra sugar. Some unchanged acetole may appear in the urine

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and so raise its total reducing power. There is an apparent rise also of the acetone bodies. The behavior of acetole in the body is explained on the basis that it dissociates into acetaldehyde and hydroxy-methylene.

Acetole is not a normal intermediate between substances of the formula  $C_6H_{12}O_6$  and those of the formula  $C_3H_6O_3$ .



## THE IODINE CONTENT OF THE THYROID AND OF SOME BRANCHIAL CLEFT ORGANS.

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(From the Department of Physiology and Physiological Chemistry,  
University of Manitoba, Winnipeg.)

(Received for publication, November 10, 1913.)

It is still a matter of uncertainty whether iodine is an invariable constituent of the thyroid gland, though all fresh evidence increases the probability that this is the case. Iodine is unquestionably present in the glands of most individuals and of most species. Numerous observers have confirmed its presence in the thyroids of man, cattle, sheep, swine, dogs, cats, and rabbits, and it has been observed further in the glands of stags, deer, goats, foxes, the stone-marten and pine-marten, guinea-pigs, hares, fowls, the African tortoise, the dogfish and skate.

In some instances negative results have been obtained. Baumann, who first observed its presence in the thyroid, obtained in man one negative case in ninety-one, in children twelve negative cases in thirty-nine, and in dogs two negative cases in nine.<sup>1</sup> Miwa and Stöltzner<sup>2</sup> stated that iodine is absent from the thyroids of normal new-born children. Roos<sup>3</sup> obtained negative results in the case of three foxes, three out of four stone-martens, one of two pine-martens, one pole-cat, four of nine domestic cats, four of fourteen dogs, and three of seven pigs. Charrin and Bourcet<sup>4</sup> obtained negative results with thyroids from eighteen of thirty-two children, and attributed the absence of iodine to their pathological condition. Mendel<sup>5</sup> obtained four negative results from

<sup>1</sup> Baumann: *Zeitschr. f. physiol. Chem.*, xxii, pp. 1-17, 1896.

<sup>2</sup> Miwa and Stöltzner: *Jahrb. f. Kinderheilk.*, xlv, pp. 83-8, 1897.

<sup>3</sup> Roos: *Zeitschr. f. physiol. Chem.*, xxviii, pp. 40-59, 1899.

<sup>4</sup> Charrin and Bourcet: *Compt. rend. de l'Acad. des Sci.*, cxxx, pp. 945-8, 1900.

<sup>5</sup> Mendel: *Amer. Journ. of Physiol.*, iii, pp. 285-90, 1900.

six children. Reid Hunt and Seidell<sup>6</sup> obtained from children, Maltese kids, an Alaskan be

In considering these negative results they be taken into account. In all these cases by some variation of the method devised after fusion with sodium hydroxide, oxid subsequent dissolution, the iodine was ex or carbon disulphide, and estimated c been shown by Seidell<sup>8</sup> that this method f results, and it can therefore be inferred of iodine will frequently escape detectio Hunt and Seidell<sup>9</sup> in referring to the "ne material state explicitly that minute qu but not detectable by their analytical m *laid on any negative results so far published* *firmed by an accurate method such as that c*

In connection with the negative figure the results of Fenger,<sup>11</sup> who has employed clearly that iodine is normally present in and young of cattle, sheep, and swine, a comparable with that of thyroids in adult that similar results will be obtained with the same method is employed.

It is well recognized that there is marked content of the thyroids of individuals of e have been obtained, but that in spite of uals there is a definite relationship to c negative results quoted, even if they re slight amount of iodine, support Roos' a roids of carnivorous animals contain muc of herbivorous species. Baumann's neg

<sup>6</sup> Hunt and Seidell: Bulletin No. 47, U. S. 1908.

<sup>7</sup> Baumann and Roos: *Zeitschr. f. physiol. C*

<sup>8</sup> Seidell: *this Journal*, x, p. 95, 1911.

<sup>9</sup> Hunt and Seidell: Bulletin No. 47, U. S. note (a).

<sup>10</sup> Hunter: *this Journal*, vii, pp. 321-49, 1910

<sup>11</sup> Fenger: *ibid.*, xi, pp. 489-92; xii, pp. 55-60

were obtained after lean meat had been fed for some time, while it is now recognized that if any assimilable iodine compound be fed the iodine content of the thyroid is increased. There is strong presumptive evidence that the diet of all forms of sea-life is unusually rich in iodine, and I have recently shown that the thyroid of the dogfish (*Scyllium canicula*) contains an amount of iodine relatively greater than any previously recorded (1.16 per cent for dry material from female fish).<sup>12</sup>

It seemed desirable to extend the observations on iodine content to as many different classes of animals as possible, with the object of increasing the evidence both as to the invariable concomitance of iodine with thyroid tissue, and as to its variation in different classes consequent on their different diets. I have now obtained positive results with the thyroids of the pigeon, the alligator, the leopard frog (*Rana pipiens*) and a second species of dogfish (*Acanthias vulgaris*). The results for the pigeon are distinctly high, those for the alligator and frog distinctly low, in agreement with their respective herbivorous and carnivorous diets.

In addition some data are included bearing on the presence of iodine in parathyroid tissue. The sole results suggesting the presence of iodine in parathyroids in amounts comparable with that in thyroids are those of Gley.<sup>13</sup> He found that in rabbits the absolute amount of iodine was greater in parathyroids; in dogs the relative amount was greater. He employed the Baumann method and any error in his results must apparently be attributed to the small quantities of materials employed. Chenu and Morel<sup>14</sup> investigated dogs, rabbits, and fowls, comparing equal quantities of thyroid and parathyroid from the same animal. They concluded from their results that the parathyroid contains iodine, but to a less extent than the thyroid. Doyon and Chenu<sup>15</sup> found that the parathyroids of the African tortoise contain little or no iodine. Estes and Cecil<sup>16</sup> obtained negative results with the glands of the cow, horse, sheep, and man. Infinitesimal amounts present in

<sup>12</sup> Cameron: *Biochem. Journ.*, vii, pp. 466-70, 1913.

<sup>13</sup> Gley: *Compt. rend. de l'Acad. des Sci.*, cxxv, pp. 312-5, 1897.

<sup>14</sup> Chenu and Morel: *ibid.*, cxxxviii, pp. 1004-7, 1904.

<sup>15</sup> Doyon and Chenu: *ibid.*, cxxxix, pp. 157-8, 1904.

<sup>16</sup> Estes and Cecil: *Johns Hopkins Hospital Bulletin*, xviii, pp. 331-2, 1907.

two experiments with dogs' and one with horses' parathyroids were attributed to accidental thyroid contamination.

I have obtained absolutely negative results with the ventral branchial body of the frog—an organ whose function is still unknown. The presence of one or more parathyroids in close juxtaposition probably resulted in the removal of these organs with the ventral branchial body, so that the results may bear also on the iodine content of these organs.<sup>17</sup> I have also carried out a comparison between the parathyroids and thyroids in a series of dogs. The results show at least a marked differentiation, while the actual amount found in the parathyroids may be completely attributable to the almost unavoidable contamination with thyroid tissue incident on the removal of the internal parathyroids in the dog. The results as far as they go support Estes and Cecil's conclusions that the parathyroids do not contain iodine.

I have used Hunter's method throughout. It has been tested and found accurate for ordinary amounts of iodine by numerous observers. I found some difficulty at first in obtaining perfectly negative results in known tests where iodine was absent, but found that, where the quantities of material analyzed were not greater than 0.5 gram, and after combustion, solution, and chlorination, the not-too-acid solution was boiled vigorously for at least one and one-half hours, the quantity of liquid being kept throughout between 150 and 300 cc., blank tests invariably gave perfectly negative results. Numerous tests with known quantities of iodine proved satisfactory. Hunter claims that his method will detect and approximately measure 0.01 mgm. of iodine (0.002 per cent of 0.5 gram). An absolutely negative result probably indicates a much lower iodine content than this figure.

#### *The pigeon.*

I have found only a single observation on the iodine content of the thyroid of birds. Chenu and Morel<sup>18</sup> compared the thyroids

<sup>17</sup> A full account of the anatomical relationships of these bodies and of the thyroid, for which the ventral branchial body must frequently have been mistaken, has been published recently by Mrs. F. D. Thompson: *Phil. Trans. (B)*, cci, pp. 91-132, 1910.

<sup>18</sup> *Loc. cit.*

and parathyroids of the domestic fowl. Since they took weights of thyroid tissue equal to those of the parathyroids present, only very small quantities were employed, and the results cannot be other than inaccurate. They are furthermore expressed for fresh tissue. This does not allow easy comparison with other published data. I have found that such small amounts of tissue can be weighed accurately only with difficulty on account of rapid drying. An approximation to the dry-tissue value may perhaps be obtained by dividing their results by four.

	WEIGHT OF THYROID TAKEN	AMOUNT OF IODINE FOUND	PER CENT IODINE	
			Fresh tissue	Dry tissue
	<i>gram</i>	<i>gram</i>		
1 year old cock.....	0.019	0.000011	0.058	(0.014)
1 year old cock.....	0.026	0.000014	0.054	(0.013)

It is doubtful whether these results show even the order of the amount of iodine present.

I have carried out a series of analyses with the domestic pigeon. Material was obtained from a large number of no certain type. In most cases the pigeons were less than six months old, the thymus being still well developed. The thyroid could be dissected completely from surrounding tissue, and the results are therefore probably correct to within 1 or 2 per cent (allowing for a trace of unremoved fibrous capsule). The material was dried *in vacuo* over sulphuric acid in this and all the succeeding analyses.

NUMBER OF THYROIDS TAKEN	WEIGHT		AMOUNT OF IODINE FOUND	PER CENT IODINE IN THE DRY GLAND
	Moist	Dry		
	<i>gram</i>	<i>gram</i>	<i>gram</i>	
23 (from 12 birds).....	0.236	0.075	0.000412	0.550
47 (from 24 birds).....	0.562	0.135	0.000644	0.477
36 (from 18 birds).....	0.504	0.117	0.000530	0.453
	Total, 0.327		0.001586	Average, 0.485

*The alligator.*

Three thyroids were obtained from three young alligators each about twelve inches long. The dissection was clean, and the degree of error is determined only by the small amount of tissue examined.

WEIGHT OF DRIED THYROIDS	AMOUNT OF IODINE FOUND	PER CENT IODINE IN DRY TISSUE
gram	gram	
0.0402	0.0000239	0.059

These results agree with the low figures found generally for carnivorous animals.

*The frog (Rana pipiens).*

Treupel<sup>19</sup> injected Baumann's iodothyryin subcutaneously into frogs, and in two cases removed the thyroids (under Gaupp's direction), and, employing Baumann's method, considered that he obtained unmistakable evidence of the presence of iodine in the tissue examined. This affords no evidence as to the presence of iodine under normal conditions, although Gaupp claims that it indicates that the thyroids of the frog function as in other vertebrates. Gaupp himself<sup>20</sup> states that he has confirmed the presence of thyroid tissue in the frog (*R. esculenta*, var. *Hungarica*) by a chemical test ("Die chemische Diagnose bestätigte, dass nicht irgend etwas Anderes, Muskelfasern und dergl. fälschlich für die Schilddrüse genommen war"), and since he immediately refers to Treupel's work he presumably indicates the presence of iodine, though I have found no further details of his examination.

I have examined the thyroids and ventral branchial bodies obtained from a large number of frogs (*Rana pipiens*) bought from Chicago and Minneapolis dealers during the period September to December, 1912, so that these frogs varied from well nourished to partially nourished individuals. On account of the minute size of the thyroid in the frog it is probable that some surrounding muscular tissue was frequently removed with it.

<sup>19</sup> Treupel: *Münch. med. Wochenschr.*, xliii, pp. 885-6, 1896.

<sup>20</sup> Gaupp: cf. *Ecker-Wiedersheim's Anatomie des Frosches*, III, i, p. 206.

ORGAN	DRY WEIGHT	WEIGHT TAKEN	AMOUNT OF IODINE FOUND	PER CENT IODINE IN DRY TISSUE
	gram	gram	gram	
185 thyroids.....	0.2089	0.0987	0.0000727	0.073
		0.1102	0.0000592	0.054
		Total, 0.2089	0.0001319	Average, 0.063
188 ventral branchial bodies.....	0.2655	0.1169 0.1371	0 0	

The dry thyroid material was greasy as though some fatty tissue was present. In consequence sampling was difficult; this probably explains the non-agreement in the two results. They indicate definitely that iodine is present in the frog's thyroid under normal conditions. As has been mentioned, surrounding tissue was probably present, so that the figure must be regarded as a minimum one, to the extent perhaps of a from 20 to 40 per cent error. Even with this correction the amount present is distinctly small, corresponding with the known carnivorous habits of the frog.

*The dogfish (Acanthias vulgaris).*

As far as I am aware the only data for fish thyroids hitherto published are those I obtained this year for *Raia clavata* and *Scyllium canicula*.<sup>21</sup> The samples of *Raia* gave figures varying from 0.283–0.438 per cent. A sample of male *Scyllium* thyroids gave the figure 0.719 per cent, another of thyroids from female *Scyllium* the very high figure 1.16 per cent.

Through the kindness of Professor E. E. Prince, Dominion Commissioner of Fisheries, a consignment of *Acanthias* was obtained last winter from the Atlantic Coast. They were preserved in formalin during transit, and it was found difficult to dissect the thyroid in the preserved fish, since the tissues had become hardened and discolored. In order to be certain that thyroid tissue was present much of the surrounding tissue was frequently removed, and the figure obtained consequently only indicates the order of the amount present. It was much smaller than that found for *Scyllium*. In

<sup>21</sup> *Loc. cit.*

## Iodine Content of Thyroid

all, 0.752 gram of material (dry) was obtained from a large number of fish.

WEIGHT TAKEN	AMOUNT OF IODINE FOUND	PER CENT IODINE IN DRY TISSUE
gram	gram	
0.2015	0.000271	0.134
0.1999	0.000263	0.131
		Average, 0.133

*Comparison of the iodine content of the parathyroids and thyroids of the dog.*

Twenty-three external and ten internal parathyroids were obtained from twelve dogs. They yielded 0.077 gram dry material which contained 0.0000120 gram iodine corresponding to 0.015 per cent. The thyroids were analyzed with the following results:

WEIGHT OF DOG	WEIGHT OF THYROIDS		AMOUNT OF THYROID TAKEN	AMOUNT OF IODINE FOUND	AMOUNT OF IODINE IN WHOLE GLAND	PER CENT IODINE IN DRY TISSUE
	Fresh	Dry				
kgm.	grams	grams	gram	gram	gram	
23.0	4.916	1.276	0.505	0.0000534	0.0001349	0.
13.0	4.793	1.379	0.502	0.0007797	0.0021418	0.
16.2	2.119	0.672	0.672	0.0017798	0.0017798	0.
5.0	13.093	3.212	0.500	0.0004472	0.0028728	0.
3.0	3.977	1.119	0.500	0.0009311	0.0020638	0.
17.5	3.123	0.937	0.505	0.0005093	0.0009450	0.
20.0	6.433	1.460	0.503	0.0004009	0.0011636	0.
21.0	7.589	1.840	0.500	0.0000576	0.0002120	0.
16.5	1.178	0.372	0.372	0.0006614	0.0006614	0.
16.0	2.684	0.792	0.792	0.0005497	0.0005497	0.
17.5	1.591	0.532	0.532	0.0001822	0.0001822	0.
22.5	2.641	0.743	0.743	0.0009407	0.0009407	0.
		Total, 14.334			Total, 0.0136677	Average, 0.

The significance of this result has already been pointed out.

## SUMMARY OF RESULTS.

Iodine is present in the thyroids of the pigeon, alligator, and frog, in amounts corresponding with the diets of these animals. It is also present in the thyroid of the dogfish (*Acanthias*). Fur-



ther support is therefore given to the theory that it is an invariable constituent of thyroid tissue. All reliable data hitherto published point to this invariable concomitance. The negative figures obtained by some investigators have led to some doubt as to the bearing of the presence of this element on the function of the gland,<sup>22</sup> but it seems desirable to reject these negative figures entirely until more certain evidence is available.

Iodine is absent from the ventral branchial body of the frog.

The amount of iodine present in the parathyroids of the dog is of a less order of magnitude than that in the corresponding thyroids, if indeed the actual quantity observed be not wholly attributable to thyroid contamination. The results, so far as they go, indicate a differentiation of function between the thyroid and parathyroid.

Mrs. F. D. Thompson very kindly dissected the frog and alligator material for me, and, with Professor Vincent, dissected the dogfish. Professor Vincent dissected the dogparathyroids. My thanks are due to both for their very kind assistance in making this work possible.

The work forms part of a research conducted under the direction of the Committee on Ductless Glands of the British Association for the Advancement of Science. The expenses have been defrayed by grants from the British Association, and (to Professor Vincent) from the Government Grant Committee of the Royal Society of London.

<sup>22</sup> Swale Vincent: *Internal Secretion and the Ductless Glands* (London, Arnold), 1912, p. 312 *et. seq.*



## A GENERAL METHOD FOR THE CONVERSION OF FATTY ACIDS INTO THEIR LOWER HOMOLOGUES.

By P. A. LEVENE AND C. J. WEST.

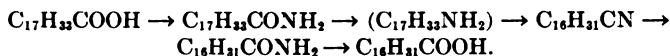
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New York.)

(Received for publication, November 17, 1913.)

Several general methods have been proposed for breaking down the carbon chain of the fatty acids, all of which are open to the objection either of poor yields or of lengthy and tedious procedure.

The first of these was developed by Krafft.<sup>1</sup> This consisted in distilling the barium salt of the  $C_n$  fatty acid with a slight excess of barium acetate in vacuum by which the methyl ketone  $C_{n-1}COCH_3$  was formed. When this was carefully oxidized with potassium dichromate and dilute sulphuric acid, acetic acid was split off and the desired acid,  $C_{n-1}H_{2n-2}O_2$ , obtained.

A second method consists in the use of Hofmann's reaction.<sup>2</sup> In this the acid is changed into the acid amide, which is treated with three molecules of bromine and eight molecules of sodium hydroxide, giving the nitrile of the next lower fatty acid. This is then easily saponified to the acid amide and then to the acid. The steps are as follows:



Still another method has been published by Le Sueur<sup>3</sup> and Blaise<sup>4</sup> (the methods are the same, the interpretation of the course of the reaction different). The  $C_n$  acid is changed into the  $\alpha$ -bromo-

<sup>1</sup> Krafft: *Ber. d. deutsch. chem. Gesellsch.*, xii, p. 1664, 1879.

<sup>2</sup> A. W. Hofmann: *ibid.*, xvii, pp. 1406 and 1920, 1884; E. Lutz: *ibid.*, xix, p. 1433, 1889.

<sup>3</sup> Le Sueur: *Journ. Chem. Soc.*, lxxxv, p. 827, 1904; lxxxvii, p. 1888, 1905.

<sup>4</sup> Blaise: *Compt. rend. de l'Acad. des. Sci.*, cxxxviii, p. 697, 1904; *Bull. soc. chim.* (3), xxxi, pp. 483-93, 1904.

and  $\alpha$ -hydroxy-derivative, from which the aldehyde of the next lower acid is prepared by heating to  $275^\circ$  for about one hour, carbon monoxide being evolved during the heating. This aldehyde is then oxidized to the acid. Since the yield of the aldehyde is not over 50 per cent this method is not perfectly satisfactory, though Le Sueur claims it to be "a ready means of passing from an acid of the acetic acid series to the next lower homologue."

In certain cases, where the halide of the  $C_{n-2}$  alcohol is easily accessible, as is cetyl iodide, the  $C_{n-1}$  acid may be synthesized from the Grignard reagent and carbon dioxide.<sup>5</sup>

Edmed<sup>6</sup> has observed that dihydroxystearic acid may be oxidized with alkaline permanganate at the place where the hydroxyls are attached. This method has been applied to the study of cerebronic acid,<sup>7</sup> where it has been shown that the principal if not the only product of the reaction is lignoceric acid. It was then concluded to apply this method of oxidation on a larger number of  $\alpha$ -hydroxy-fatty acids. It is comparatively an easy task to transform a fatty acid into its  $\alpha$ -hydroxy-acid. If the permanganate method of oxidation were successful we hoped to apply the process for the study of the structure of lignoceric acid and of other fatty acids, the structure of which is not yet definitely established. Considerable time after the publication of the work on cerebronic acid Lapworth<sup>8</sup> made use of the same process for the preparation of tridecylic from myristic acid. Since then we have had occasion to prepare a considerable quantity of margaric acid and have applied the reaction to  $\alpha$ -hydroxystearic acid with equal success. It has further been tried out in the preparation of pentadecylic acid and we now believe that it is a general method for the decomposition of the carbon chain which may be easily carried out with fairly good yields. We obtained a yield of 80–85 per cent of margaric acid, calculated on the stearic acid used. It has the advantage over Le Sueur's method in that the preparation of the aldehyde with its consequent loss is avoided.

<sup>5</sup> Ruttan: *Eighth International Congress of Applied Chemistry*, xxv, p. 431; *Chem. Abstracts*, vii, p. 2140, 1913.

<sup>6</sup> Edmed: *Journ. Chem. Soc.*, lxxiii, p. 627, 1898.

<sup>7</sup> Levene and Jacobs: this *Journal*, xii, p. 381, 1912; Levene and West: *ibid.*, xiv, p. 257, 1913.

<sup>8</sup> Lapworth: *Journ. Chem. Soc.*, ciii, p. 1029, 1913.

In agreement with Lapworth we find that the reaction is best carried out in acetone rather than in water. The potassium salt of the new fatty acid (especially of the higher acids) is insoluble in acetone and precipitates with the manganese dioxide, from which it is easily extracted with alcohol.

#### EXPERIMENTAL PART.

##### *Lignoceric acid.*

The preparation of lignoceric acid has been modified to the following: Fifty grams of cerebronic acid are dissolved in about 1 liter of boiling acetone and this solution treated gradually with a warm acetone solution of potassium permanganate until the solution is slightly colored, indicating an excess of permanganate. The mixture is then heated a short time on the water bath, cooled, the manganese dioxide and potassium lignocerate and cerobronate filtered off, and the potassium salt extracted with boiling absolute alcohol. Usually two or three extractions, using a liter of alcohol each time, is sufficient. The acid thus obtained is converted at once into the lithium salt and purified with methyl alcohol as before. The acid from the insoluble lithium salt was recrystallized from acetone, when it gave the following numbers on analysis:

0.1232 gram of substance gave 0.3537 gram  $\text{CO}_2$  and 0.1460 gram  $\text{H}_2\text{O}$ .

	Calculated for $\text{C}_{24}\text{H}_{48}\text{O}_2$	Found:
C .....	78.20	78.30
H .....	13.20	13.26

##### *Margaric acid.*

Stearic acid was converted into  $\alpha$ -bromostearic acid by Hell's method and this into  $\alpha$ -hydroxystearic acid according to Le Sueur. This was characterized by the preparation of

##### *$\alpha$ -Acetoxystearic acid.*

Fifteen grams of hydroxystearic acid were dissolved in 100 grams of acetyl chloride and the solution heated an hour in a water bath. The excess of acetyl chloride was removed on a boiling water bath, the product treated with water to remove the last

traces of the chloride and hydrochloric acid, extracted with ether, the ethereal solution dried, the ether removed and the product recrystallized from a little absolute alcohol. It is a colorless crystalline body which melts at 70–70.5°.

0.1258 gram of the substance gave 0.3243 gram  $\text{CO}_2$  and 0.1258 gram  $\text{H}_2\text{O}$ .

	Calculated for $\text{C}_{15}\text{H}_{31}\text{O}_2$ :	Found:
C.....	70.12	70.31
H.....	11.19	11.19

Oxidized with potassium permanganate in acetone as given above, the hydroxystearic acid gave nearly pure margaric acid, which was purified by two recrystallizations out of acetone. It melted at 59–60°, as given by Ruttan.<sup>9</sup> Its purity was controlled by analysis:

0.1228 gram of the substance gave 0.3386 gram  $\text{CO}_2$  and 0.1360 gram  $\text{H}_2\text{O}$ .

1.0000 gram of the acid, dissolved in absolute alcohol and benzene required 37.0 cc.  $\frac{N}{10}$  NaOH for neutralization, using phenolphthalein as an indicator.

	Calculated for $\text{C}_{17}\text{H}_{33}\text{O}_2$ :	Found:
C.....	75.60	75.20
H.....	12.60	12.40
M.W.....	270	270

#### *Pentadecylic acid.*

Pentadecylic acid was prepared in the same manner, starting with palmitic acid. The acid thus prepared melted at 53° and gave the following analytical figures.

I. 0.1342 gram of the substance gave 0.3674 gram  $\text{CO}_2$  ( $\text{H}_2\text{O}$  lost).

II. 0.1324 gram of the substance gave 0.3620 gram  $\text{CO}_2$  and 0.1472 gram  $\text{H}_2\text{O}$ .

1.0000 gram of the acid, as above, required 41.4 cc.  $\frac{N}{10}$  NaOH for neutralization.

	Calculated for $\text{C}_{15}\text{H}_{31}\text{O}_2$ :	Found:	
		I	II
C.....	74.40	74.67	74.57
H.....	12.40		12.44
M.W.....	242	241	

<sup>9</sup> Ruttan: *loc. cit.*

## AUTOLYSIS OF MOLD CULTURES II.

### INFLUENCE OF EXHAUSTION OF THE MEDIUM UPON THE RATE OF AUTOLYSIS OF *ASPERGILLUS NIGER*.

By ARTHUR W. DOX.

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(Received for publication, November 17, 1913.)

In a previous paper<sup>1</sup> it was shown that when molds are grown upon a fluid synthetic medium, the nitrogen is almost completely taken up by the mycelium during the vegetative period of the fungus and then gradually returned to the medium after the growth has come to a standstill. In the particular medium studied where nitrogen and sucrose were present in the proportion of 1 to 50, most of the nitrogen had disappeared at the end of the first week, and during the subsequent seven or eight weeks a large part of it reappeared, principally in the form of ammonium salts. During this time the mycelium lost its turgidity and the medium became dark in color although it retained its original clarity. This phenomenon was ascribed to autolysis of the fungus.

The changes observed both in the mycelium and in the medium were so striking as to be deemed worthy of further study. Subsequent observations of numerous cultures showed that the rate of autolysis, as indicated by the visual appearance of the mycelium and of the medium, was influenced by a number of factors, such as the volume of the medium as compared with the surface area, the ratio of carbon to nitrogen, the temperature to which the cultures were exposed, etc. In other words, the supply of nutriment and the rate of growth seemed to be of primary importance in determining the point at which autolysis set in. As long as the presence of sugar could be demonstrated by Fehling's test, autolysis was scarcely noticeable.

<sup>1</sup> Dox and Maynard: this *Journal*, xii, pp. 227-31, 1912.

The experiments herein described were made for the purpose of studying the effect of exhaustion of the carbohydrate in the medium upon the autolysis of the fungus.

In the first series of cultures the following medium was used: distilled water 1000 cc., sucrose 30 grams, dibasic potassium phosphate 1.0 gram, magnesium sulphate 0.3 gram, ammonium acid tartrate 4.0 grams, trace of ferrous sulphate. Two hundred cc. of this medium were placed in each of a number of liter Erlenmeyer flasks, sterilized in an autoclave, and inoculated with spores of *Aspergillus niger*. At the end of a week vigorous cultures with an abundance of black spores were obtained. Two of the cultures were treated as follows: The cotton plug was removed, a sterile glass siphon inserted and the plug replaced. The medium was drawn off by suction, replaced by 200 cc. of sterile distilled water, and the latter removed in the same way after a few moments' contact with the mycelium. This was in turn replaced by 200 cc. of sterile water in one flask and by 200 cc. of a sterile 2 per cent sucrose solution in the other flask, and the cultures allowed to stand another week. This operation was repeated at the end of each week for six successive weeks. All this was done with as little injury as possible to the mycelium, care being taken not to wet the surface of the culture. Five other flasks were treated in the same manner after the cultures had grown two, three, four, five and six weeks respectively, except that the cultures were discarded after the medium and wash water had been obtained. The combined

TABLE I.

AGE OF CULTURE	a.		b.		c.
	MEDIUM REPLACED WEEKLY BY WATER		MEDIUM REPLACED WEEKLY BY 2% SUCROSE		MEDIUM FROM UNDISTURBED CULTURE
	mgm. N in medium		mgm. N. in medium		mgm. N
weeks		total		total	
0	70.0		70.0		70.0
1	2.7	2.7	2.5	2.5	2.4
2	12.4	15.1	3.1	5.6	9.4
3	12.8	27.9	2.3	7.9	16.9
4	5.9	33.8	1.4	9.3	23.5
5	3.6	37.4	1.0	10.3	26.1
6	3.0	40.4	1.7	12.0	27.3



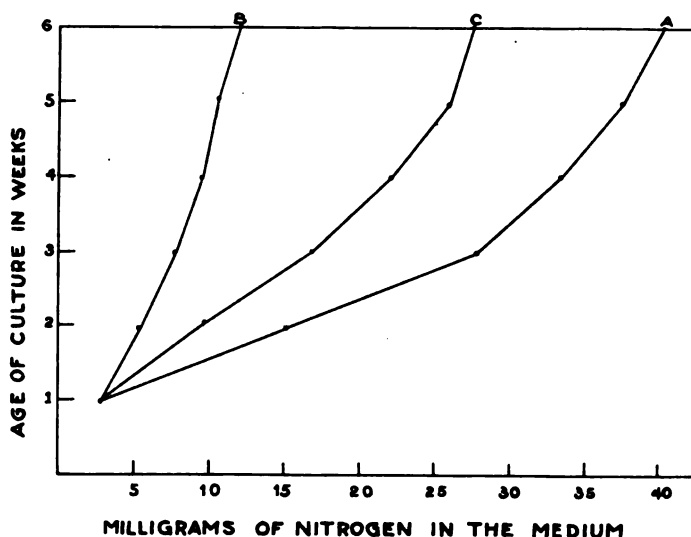


FIG. 1.

medium and wash water were used in each case for the determination of total nitrogen by the Kjeldahl method. As the liquid was not filtered it contained a few of the submerged mycelial filaments, but the nitrogen content of these was so small as to be negligible. This method of obtaining the medium does not allow for the liquid still retained between the closely matted hyphae, but no better method presented itself which would not be apt to injure the mycelium and disturb the normal progress of growth and autolysis of the organism. The results of the nitrogen determinations are given above.

The above experiments were repeated using Raulin's well-known medium, on which this organism grows still more luxuriantly. At the end of a week dense white mycelia were obtained, with spores just beginning to show around the edges. An abundance of black spores appeared about two days later. The sucrose solution used in this series was 4.67 per cent corresponding to the concentration of the same in Raulin's medium.

In both series the nitrogen restored to the medium in six weeks is more than three times as great when the medium is replaced weekly by distilled water as when it is replaced by sucrose solution.

TABLE II.

AGE OF CULTURE	MEDIUM REPLACED WEEKLY BY WATER		MEDIUM REPLACED WEEKLY BY 2% SUCROSE	
	mgm. N in medium		mgm. N in medium	
weeks		total		total
0	225.3		225.3	
1	17.5	17.5	18.3	18.3
2	48.2	65.7	8.3	26.6
3	46.2	111.9	8.5	35.1
4	20.0	131.9	4.9	40.0
5	14.3	146.2	4.5	44.5
6	4.5	150.7	3.8	48.3

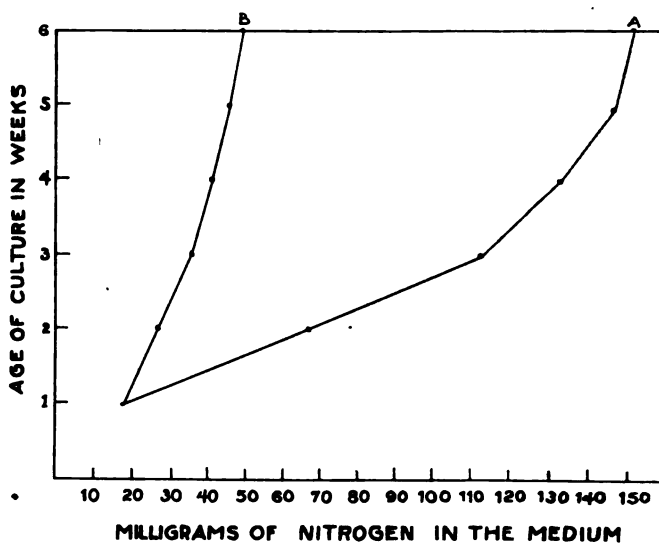


FIG. 2.

Other differences are equally striking. In the one case the medium is dark brown in color and neutral to litmus each time it is drawn off, in the other case it is pale yellow and strongly acid. The mycelium on water becomes thin and limp, while that on sucrose becomes more and more dense and retains its turgidity. It is evident that vegetative growth continues for a much longer period when sugar is supplied from time to time, although no nitrogen or inorganic

salts are added. Even after autolysis has been in progress for five weeks, the substitution of sucrose solution for the dark colored medium results in a marked decrease in the amount of nitrogen liberated. During the sixth week a five-week-old culture that had not previously been disturbed liberated only 10.4 mgm. nitrogen into the sucrose solution. A parallel culture liberated 48.2 mgm. nitrogen into distilled water.

The first series (Table 1) shows that the rate of autolysis is increased by removing the products of autolysis, as will readily be seen by comparing column *c* with the totals in column *a*. Where the autolytic products are not removed, the nitrogen determinations take a position on the curve intermediate between those occupied by the nitrogen in the sucrose medium and the nitrogen in the water.

The color of the medium may be taken as an indication of the extent of autolysis. Invariably the intensity of the brown color was proportional to the amount of nitrogen in solution.

When the medium is replaced from week to week by Raulin's medium instead of the sucrose solution, the greater part of the nitrogen continues to be assimilated, and the resulting mycelium is even more dense than that from sucrose alone. At the end of six weeks the mold was removed only with difficulty from the flask. On the other hand, the addition of an antiseptic appears to increase the rate of autolysis. A culture one week old, when floated upon 200 cc. of a dilute solution of mercuric chloride ( $\frac{M}{1000}$ ), yielded 136.2 mgm. of soluble nitrogen as compared with 48.8 mgm. and 8.3 mgm. in the parallel experiments with water and sucrose respectively. However, the effect of the antiseptic after spore production is much less pronounced.

During autolysis the weight of the mycelium decreases while the percentage of nitrogen in the latter remains fairly constant. This is clearly indicated by the following data, where the mycelium from five cultures of *Aspergillus fumigatus* was removed each week, pressed as free as possible from the medium and dried in an oven.

The weights in the following table were not expressed more accurately for the reason that the autolyzed mycelium becomes pasty when subjected to pressure and cannot be removed completely from the cloth. The probable loss from this cause was about 1 per cent. The data are sufficiently accurate, however, to show

TABLE III.

AGE OF CULTURE	DRY WEIGHT OF MYCELIUM	NITROGEN IN MYCELIUM	
		per cent	gram
weeks	grams		
3	11.5	6.14	0.706
4	10.0	6.24	0.624
5	9.0	5.78	0.520
6	8.5	5.73	0.487
7	7.9	5.68	0.500
8	7.5	6.08	0.456
9	7.4	5.62	0.416
10	7.0	5.52	0.386
13	6.5	5.66	0.368

the gradual loss in weight when the mycelium undergoes autolysis, while the percentage of nitrogen scarcely changes. The weights for the first and second weeks were not obtained, but it is probable that the loss at the end of thirteen weeks is at least 50 per cent of the maximum weight on the dry basis.

The more important observations recorded in this paper may be summarized as follows:

1. Autolysis of cultures of *Aspergillus niger* is due chiefly to exhaustion of carbohydrate from the culture medium.
2. The rate of autolysis is increased by removing the autolytic products and replacing by distilled water.
3. Replacement of the medium at regular intervals by a sucrose solution reduces the rate of autolysis to less than half that of the undisturbed culture, and less than one-third that of the cultures where the medium is replaced by distilled water.
4. Autolysis is attended by a loss in weight of the mycelium amounting to about 50 per cent in thirteen weeks.

My thanks are due to Mr. W. G. Gaessler who kindly made the nitrogen determinations.

## CARBON DIOXIDE APPARATUS III.<sup>1</sup>

### ANOTHER SPECIAL APPARATUS FOR THE ESTIMATION OF VERY MINUTE QUANTITIES OF CARBON DIOXIDE.

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(Received for publication, November 22, 1913.)

For the purpose of estimating a very small amount of carbon dioxide in general biological problems, two pieces of apparatus have been already described.<sup>2</sup> The principle of the apparatus is as follows:

1. Exceedingly minute quantities of carbon dioxide can be precipitated as barium carbonate on the surface of a small drop of barium hydroxide solution.

2. When a drop of barium hydroxide is exposed to any sample of gas free from carbon dioxide it remains perfectly clear, but when more than a quite definite minimum amount of carbon dioxide is introduced a precipitate of carbonate appears, detectable with a lens.

3. By determining, therefore, the minimum volume of any given sample of a gas necessary to give the first visible formation of the precipitate, its carbon dioxide content can be estimated accurately, since this volume must contain just the known detectable amount of carbon dioxide.

In order to determine this minimum volume of the gas in the respiratory chamber, it has been recommended that in the case of biological problems, when the specimen gives off carbon dioxide continuously, and sometimes at different rates, varying with the time, it would be much simpler not to attempt to determine the minimum

<sup>1</sup> This and my other apparatus can be obtained from Eimer and Amend, New York.

<sup>2</sup> *Amer. Journ. of Physiol.*, xxxii, p. 137, 1913.

volume by a continuous trial with the same sample of tissue, but instead to repeat the experiments with a series of different samples of known weights for a known time, and determine the minimum volumes which give the precipitates, and the maximum volumes which do not give the precipitates.<sup>3</sup> In this way, one can easily calculate what is the minimum volume which gives the precipitate, for a given weight of the specimen for a given time. All of the analyses of the gas in connection with metabolic problems of the nerve fiber have been done in this way with satisfactory accuracy.

Although the use of the biometer (apparatus II) is perfectly satisfactory for almost all micrometabolic problems, and sometimes absolutely necessary for quick quantitative comparisons between two different tissues, yet it is sometimes inconvenient for a complete determination of the CO<sub>2</sub>-production from a single tissue, the metabolic rate of which is constantly changing, and the available amount of which is not very great. The necessity for a device to prevent this difficulty was keenly felt when I was studying the metabolism before, after and during the cleavage of a single fertilized egg this summer. The new apparatus III, here reported, proved to be indispensable for such an investigation and may be of some interest for general biological problems, for which the previous apparatuses are found to be useful.

The new feature of this apparatus III is a device by which the air can be withdrawn into a tube from the respiratory chamber and can be analyzed subsequently. With this device, one can not only make a complete analysis with one sample of the tissue, but can also make several complete estimations with it. The detailed method will make this clear.

### 1. The apparatus.

As shown in figure 1, the main part of this apparatus consists of only one glass bulb *A*, which serves the combined purpose of respiratory and analytic chambers. Its volume, originally 30–40 cc., can be diminished by introducing mercury in exactly the same manner as described previously. Similarly, the Ba(OH)<sub>2</sub> tube *d* is inserted through its wall, and a three-way stopcock 4 is attached to the bottom of the chamber. Just opposite the top of

<sup>3</sup> See a footnote on p. 140, *loc. cit.*

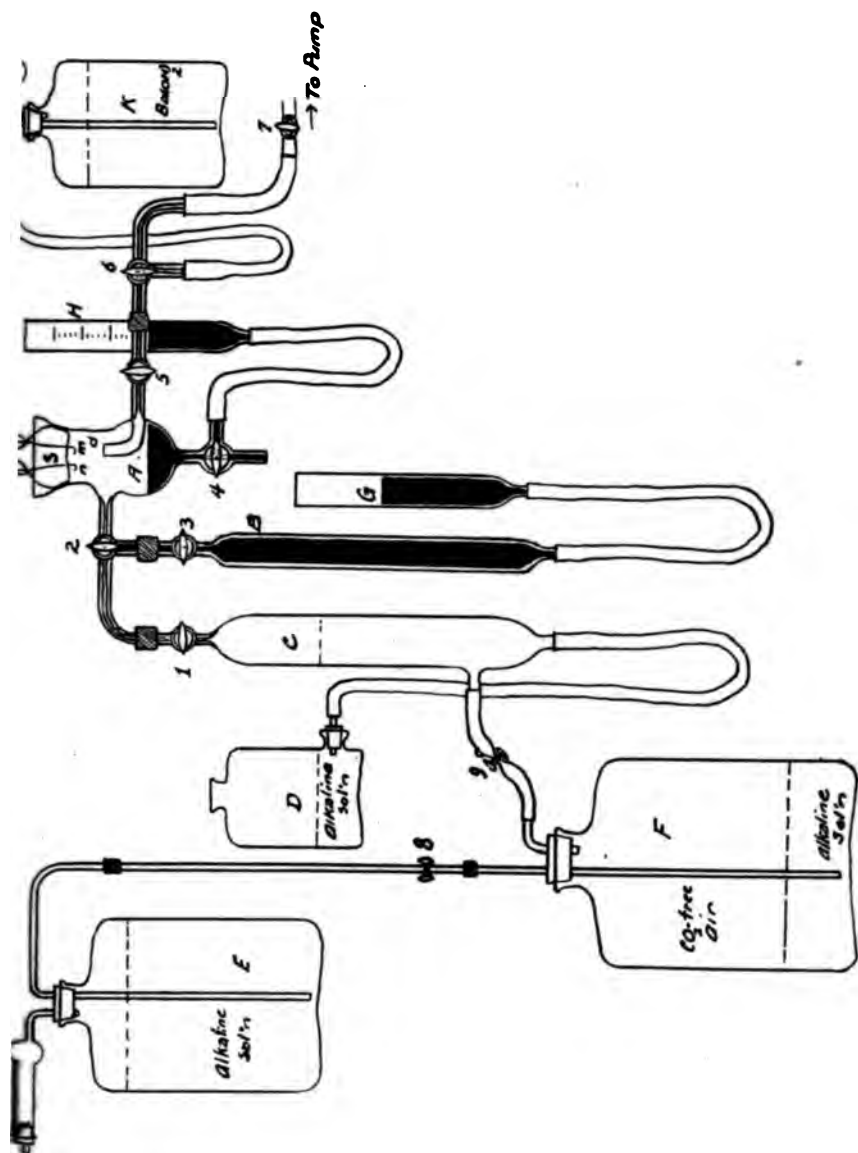


FIG. 1. A simpler apparatus for  $\text{CO}_2$  analysis, showing also the device to obtain air free from  $\text{CO}_2$ ; † actual size except E and F which are 1/2.

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the  $\text{Ba}(\text{OH})_2$  tube *d*, another three-way stopcock 2 is inserted, one arm of which is connected to the nitrometer *C*, and the other to tube *B*. This tube *B* is attached to the mercury burette *G*, by which the pressure in the tube is to be adjusted. A similar mercury burette is also attached to one arm of three-way stopcock 4 for the same purpose.<sup>4</sup>

### 2. How to obtain $\text{CO}_2$ -free air.

Several inquiries have been made as to the exact arrangements for obtaining  $\text{CO}_2$ -free air, and how to use them. As I have stated elsewhere, it is very difficult to make air completely free from carbon dioxide by merely passing it through alkaline wash bottles. A simpler and sure method to obtain  $\text{CO}_2$ -free air is shown in fig. 1. It is prepared by shaking air with a 20 per cent solution of sodium hydroxide in a tightly stoppered carboy *F*, supplied with suitable tubes. When this air is to be used it is driven into a nitrometer *C*, which is filled with less concentrated alkaline solution (a weak solution is used so that the chamber may not be too dry), by displacing it by running into *F* a solution of sodium hydroxide with a funnel, or from another carboy *E* which is filled with the alkali. After each evacuation of the chamber, this air is introduced from the nitrometer *C* into the chamber *A* through stopcock 1. For ordinary experiments, one can always keep the pressure in *F* high enough so that  $\text{CO}_2$ -free air may be driven several successive times into nitrometer *C* by simply opening pinchcock 9.

In order to test whether or not the air thus treated is now free from carbon dioxide, the following experiment will be necessary.

Remove the glass stopper *S*, and introduce into the chamber a known amount of mercury by means of the mercury burette *H*, so that the remaining volume of the chamber *A* is exactly known.<sup>5</sup>

<sup>4</sup> Instead of fusing the platinum electrodes into the wall of the chamber, they were fused into the glass stopper *S*. It will be clear by inspection of the figure that the wires are brought up high enough so that when the chamber is sealed with mercury there will be no short circuit established through mercury and electrodes, in case any electric current is used for the stimulation of the tissue.

<sup>5</sup> The exact capacity of chamber *A* should be calibrated once for all. In this way, one can always work with a constant volume in the chamber by



Turn stopcock 4 now about  $45^\circ$ , so that the connection between *A* and *H* is severed. Replace the stopper *S*, and seal the chamber with mercury. Keep stopcock 2 turned so that the connection is made only between nitrometer *C* and chamber *A*. The alkali in the nitrometer *C* is displaced by  $\text{CO}_2$ -free air by opening pinchcock 9. Collect about 300 cc. of the  $\text{CO}_2$ -free air in the nitrometer *C*. While the stopcock 1 is closed, the chamber *A* is evacuated by means of suction, having the stopcocks 5, 6 and 7 opened (the three-way stopcock 6 should be opened in such a way that  $\text{Ba}(\text{OH})_2$  is completely shut off from the connection).

When the evacuation is complete,  $\text{CO}_2$ -free air is introduced into the chamber by opening stopcock 1. After the evacuation and washing out with pure air, which is repeated four or five times, the chamber now being filled with  $\text{CO}_2$ -free air, the stopcock 7 is closed, and the pressure inside chamber *A* is made equal to the atmospheric pressure by adjusting it at the nitrometer *C* by means of the alkali bottle *D*. Stopcock 5 is then closed, and the space between 5 and 7 is again evacuated so that the barium hydroxide can rush in, a process which is very advantageous in obtaining a clear barium hydroxide solution. In filling the tube with the barium hydroxide, it is advisable to open stopcock 6 so that the solution will first fill up the space between 6 and 7, then turn it in such a way that now the connection is made between the barium hydroxide tube and the space between stopcocks 5 and 6. By opening 5 very slowly and carefully, the barium hydroxide is now introduced into the chamber just so far that a small hemispherical drop stands upon the upturned end of the tube at *d*. After quickly readjusting the pressure by means of the nitrometer and the bottle *D*, the stopcock 1 is closed. If the air is completely free from carbon dioxide, the drop should be clear not only at the start, but also, after several hours' standing, free from any granules of the carbonate, when inspected with the lens.

introducing the necessary odd cubic centimeters of mercury, thus making the remaining volume a convenient round number of cubic centimeters. For instance, the apparatus I am using has a capacity of 31.4 cc. I introduced 6.4 cc. of mercury for each experiment, so that the respiratory chamber then contains 25 cc. It will be needless to say that for an experiment to test the air for its purity, the knowledge of the exact capacity of the chamber is not at all necessary.

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### 3. *For the qualitative detection of carbon dioxide.*

For the detection of carbon dioxide production from a tissue, this apparatus can be used in exactly the same manner as the previous apparatus. After insuring that the air is free from the gas a given tissue<sup>6</sup> is placed inside of the chamber and the process is repeated as before. If any  $\text{CO}_2$  is given off by the tissue not only will a deposit of carbonate appear, but it will also grow in size.

### 4. *For quantitative estimation of the gas.*

The detailed method is as follows.

Open stopcocks 3 and 2 so that they will connect the chamber *A* and the tube *B* only. Fill the mercury burette *G* and raise it till the mercury will completely fill the tube *B* and a little excess of it will stay in the capillary tube between the chamber *A* and the stopcock 2. This stopcock 2 is now closed so that it will connect the nitrometer *C* and the chamber *A* only. Increase the pressure inside the nitrometer *C* by raising the alkali bottle *D* much higher than the meniscus of the alkali in the nitrometer *C* and then open stopcock 1. In this way, the excess of mercury left in the capillary tube will be pushed over into the chamber and will flow through the stopcock 4 into a receiving vessel.

If the stopcock 2 is absolutely air-tight, having no air bubble in tube 3, then a known amount of mercury is introduced into the chamber by means of the mercury burette *H*, thus giving the respiratory chamber the desired volume. The tissue is introduced into the chamber, the glass stopper is replaced, the chamber is sealed with mercury, and the nitrometer *C* is filled with the pure air. After evacuation of the chamber and washing it with  $\text{CO}_2$ -free air several times, the stopcock 5 is closed and the time is recorded; the pressure is adjusted, and stopcock 2 is turned  $45^\circ$ .

At the end of the desired respiration period, any portion of the air in the chamber can be driven into the tube *B*. This is accomplished by raising the right hand mercury burette *H*, and simultaneously opening the stopcocks 2 and 4. Stopcock 2 is now

<sup>6</sup> The tissue can be placed on a cover slide and allowed to float on top of the mercury or it can be placed on the glass plate and hung on the electrodes as described in figure 1, p. 120, *loc. cit.*

closed: the pressure of the air in *B* is kept equal to the atmospheric pressure by adjusting the mercury burette *G*. After removing the mercury seal and glass stopper *S*, the tissue is withdrawn and mercury burette *H* is lowered so that most of the mercury in the chamber will now flow back into the burette *H*. The excess of mercury in the chamber *A* is withdrawn through stopcock 4 into a vessel.

In order to analyze the air in the tube *B*, it is advisable to clean the whole chamber *A* once more with water,<sup>7</sup> and then to perform the experiment in exactly the same manner as we described in connection with the test for purity of air.<sup>8</sup> Two things are imperative, namely; the capacity of the respiratory chamber *A* must be known exactly after the known amount of mercury is introduced into it; and the bubble of barium hydroxide solution at *d* must be perfectly clear at the start. If no deposit of barium carbonate forms on the surface of the drop within ten or fifteen minutes, it is a sure control that the apparatus is free from carbon dioxide. This point established, a portion of the sample of gas in the tube *B* is introduced into the chamber. This is very easily accomplished by withdrawing the mercury from the chamber *A* into a small graduated cylinder<sup>9</sup> and adjusting the pressure by raising the left-hand mercury burette *G*; then close the stopcock 2 by turning it 45°.

One now watches the surface of the drop at *d* with a lens to see whether any formation of barium carbonate occurs within ten minutes. If it does not, we should introduce more air from *B* until we get the first visible precipitate.<sup>10</sup> I have previously determined,<sup>11</sup> by introducing accurately known quantities of carbon dioxide of very high dilution into the chamber in a similar manner, and have found with remarkable regularity that  $1 \times 10^{-7}$  gram of carbon dioxide is necessary as the minimum amount to give a

<sup>7</sup> For the method of cleaning this apparatus and drying it in ten minutes without taking it apart, see a footnote on page 138, *Amer. Journ. of Physiol.*, xxxii, 1913.

<sup>8</sup> See pages 488-89.

<sup>9</sup> If more accurate measurement is necessary, the mercury withdrawn should be weighed.

<sup>10</sup> The detection of this precipitate is not a question of degree, but is a question of the appearance of some precipitate or none at all; therefore the end point is very sharp.

<sup>11</sup> See p. 144, *loc. cit.*

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precipitate within ten minutes.<sup>12</sup> Smaller amounts of the gas give no visible results, while larger amounts give a deposit more rapidly and in larger quantities. This minimum detectable amount of  $1 \times 10^{-7}$  gram is about the amount which is contained in 0.17 cc. of natural air in which we assume 3 parts of carbon dioxide in 10,000 by volume. The following example will illustrate the calculation of the exact amount of the gas a tissue gives off.

The original volume of the respiratory chamber is 31.4 cc., to which 6.4 cc. of mercury are introduced, making the remaining volume exactly 25 cc. 10 mgms. of tissue are used and are allowed to respire in the chamber for ten minutes. Then about 10-15 cc. of the gas are withdrawn into the tube *B*. 0.5 cc. of this gas gave no precipitate during the first ten minutes; 0.5 cc. more of the sample gave no deposit in another interval of ten minutes; 0.5 cc. more, a total of 1.5 cc., was run into the chamber. A marked evidence of a precipitate appeared in five minutes. 1.5 cc. of this gas must therefore contain  $1 \times 10^{-7}$  gram of carbon dioxide. The apparatus is then cleaned and dried and a clear drop of barium hydroxide is again introduced upon the top of the tube *d*; and after again insuring the fact that the air is free from any CO<sub>2</sub>, by waiting, 1 cc. of the sample gas which has been left undisturbed in tube *B* is introduced into the chamber; no precipitate was formed within ten minutes; 0.25 cc. more of the sample did not produce any precipitate; but when 0.25 cc. more is taken, crystals of barium carbonate now appeared after a few minutes. 1.5 cc. of the gas must contain, therefore,  $1 \times 10^{-7}$  gram of carbon dioxide.

From these duplicates, it becomes certain that 1.5 cc. out of 25 cc. of the chamber now contain  $1 \times 10^{-7}$  gram of carbon dioxide. Therefore the total amount of carbon dioxide produced by 10 mgms. of the tissue during ten minutes will be

$$1 \times 10^{-7} \text{ gram} \times \frac{25}{1.5} = 16.6 \times 10^{-7} \text{ gram of carbon dioxide}^{13}$$

### 5. For a rapid collection of air for a later analysis.

With this new apparatus one can also collect the air very rapidly and analyze it at leisure, thus enabling him to collect the air at successive short intervals of respiration by the same tissue or similar tissues at different stages of activity. For this purpose, a very simple special form of the gas pipette was devised. Figure

<sup>12</sup> This is the case when the analytic chamber has about 15-20 cc. It may take a longer time to produce a precipitate, when the chamber is much larger than this.

<sup>13</sup> For correction for temperature and pressure, see footnote on p. 494.

2 will illustrate the exact shape of the tube. Instead of tube *B*, this tube is connected to the arm of the stopcock 2 at *a* by means of rubber tubing,<sup>14</sup> and the tube is connected to the mercury burette *G* at *b*.

With this arrangement, we should repeat the experiment exactly the same way as above, except that when we drive the air from the chamber to this tube, we should drive it so far as to push a few cubic centimeters of mercury also from the chamber, so that the mercury will remain in the U-tube *U*, thus automatically sealing

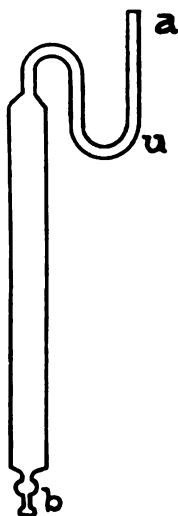


FIG. 2. A special gas pipette  $\frac{1}{2}$  actual size.

the tube. By clamping the rubber connection at *b*, this tube is removed,<sup>15</sup> and another pipette is connected, and the experiment is repeated with the same tissue or another tissue as the case may be. By this method, one can collect twenty or thirty samples of the gas a day with a single apparatus.

<sup>14</sup> Use of rubber tubing is harmless, provided the mercury burette *G* is kept above the level of stopcock 2.

<sup>15</sup> Since this pipette has a capacity of 10–20 cc., and only about 10–15 cc. of air are introduced, there will usually be 4–5 cc. of mercury left at the lower end of the tube, thus sealing it automatically at both ends. It is obvious that one should keep the tube vertical, in order to keep it air-tight.

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When we are ready to analyze the gas from these tubes, all we have to do is to connect the tube to the usual place (at 2) and raise the mercury burette *G* in such a way that the mercury in the U-tube is now driven up to fill all the capillary tubes between the chamber and the pipette, thus forcing all the atmospheric air out of the tube, and then stopcock 2 is turned so as to cut all connections. The air in the tube is then examined according to the method described before.<sup>16</sup>

<sup>16</sup> One disadvantage of this new apparatus is that we must take into consideration temperature and pressure variation, which was entirely unnecessary for the previous apparatus. If the respiration and analysis were done at different temperature and pressure, the ratio between the minimum volume which gives the first precipitate and original volume of the chamber will not be rigid. In that case, the minimum volume should be translated to the volume at the temperature and pressure at the time of respiration. Such correction, however, will not be necessary if the analysis is done immediately after the respiration, during which the variation in temperature and pressure will not affect the result beyond the experimental error, as is shown in the following calculation:

Let us suppose that 10 mgms. of tissue respire for ten minutes at 18° at 760 mm. of pressure in 25 cc. of the chamber, and suppose 1.5 cc. of the same air at 22°, at 730 mm. of pressure (making a liberal estimate of the change in temperature and pressure) gave the first precipitate; then we will obtain the following results:

a. Without any correction, we get  $1 \times 10^{-7}$  gram  $\times \frac{25}{1.5} = 16.6 \times 10^{-7}$  gram.

b. With the correction,  $1 \times 10^{-7}$  gm.  $\times \frac{25}{1.5 \times \frac{(270+18) \times 730}{(270+22) \times 760}} = 17.6 \times 10^{-7}$  gm.

This is a little over 5 per cent error, which will be the maximum, and almost an impossible variation for ordinary weather in the laboratory for a short interval of time. Besides, we are dealing with a very small sample of moist tissue, the weight of which may easily vary within 5 per cent.

## ON THE RATE OF ABSORPTION OF CHOLESTEROL FROM THE DIGESTIVE TRACT OF RABBITS.<sup>1</sup>

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(Received for publication, November 24, 1913.)

Our knowledge of the physiology of cholesterol, particularly of its absorption from food, has only in recent years made notable progress. The fact that it is, in truth, absorbed from the food, according to the uniform evidence of several investigators, may now be considered established.

The following studies were undertaken in order to determine, as accurately as possible, the rate of absorption in normal rabbits, as a basis for further investigations of the alterations of this rate in those pathological conditions, notably lipemia, in which the cholesterol metabolism is known to be greatly disturbed.

As early as 1867, Tolmatschek<sup>3</sup> sought to prove the absorption of cholesterol from the food by estimating the intake and output of cholesterol in the breast-fed child. In 1890, Thomas,<sup>4</sup> working on dogs with experimental biliary fistula, found no increase of cholesterol in the bile with a diet rich in cholesterol. Jankau,<sup>5</sup> two years later, found no increase in the feces of rabbits and dogs after a single feeding of cholesterol; but, six hours after feeding, he found also no increase in cholesterol in blood and bile and liver-substance. With this contradictory evidence he was forced to leave the question still undecided.

In 1906, the first work of real significance appeared. Pribram<sup>6</sup> fed rabbits through a stomach-tube on three successive days with the palmitic

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<sup>1</sup> These studies were made under the direction of Prof. P. Morawitz, to whom I wish here to express my gratitude, not only for valuable advice and assistance, but also for the opportunity to carry on the investigation.

<sup>2</sup> John Harvard Fellow, Harvard Medical School.

<sup>3</sup> Tolmatschek: *Hoppe-Seyler's Med.-chem. Untersuchungen*, 1867, p. 272; cited from *Oppenheimer's Handbuch der Biochemie*, 1908, Vol. IV, No. 1, p. 485.

<sup>4</sup> Thomas: *Inaug. Diss.*, Strassburg, 1890.

<sup>5</sup> Jankau: *Arch. f. exp. Path. u. Pharm.*, xxix, p. 237, 1892.

<sup>6</sup> Pribram: *Biochem. Zeitschr.*, i, p. 413, 1906.

and oleic acid esters of cholesterol and with cholesterol itself, in amounts of 1 gram on each day. One or two days later, the animal was killed and the blood and organs were analyzed for cholesterol by saponification, ether extraction and weighing. He found with feeding of esters and pure substance alike, an increase in the blood and no definite reliable results from the tissue estimations. He also showed that the serum of an animal fed with cholesterol would prevent or delay in small doses the haemolytic action of saponin on normal red blood corpuscles.

Morgenroth and Reicher<sup>7</sup> reported in the next year, the following experiments with a series of rabbits. Rabbit A was fed 4 grams of cholesterol in 15 cc. of olive oil daily, rabbit B, the same amount of pure olive oil, rabbit C, nothing beyond the ordinary diet; all three were then injected alike with lecithide. After five days, the haemoglobin estimations were respectively 58, 20, and 30 per cent; and the cholesterol percentage in the blood, respectively 0.48, 0.03, and 0.026. The three sera showed corresponding influences on saponin haemolysis *in vitro*. These results confirm Pribram.

Goodman,<sup>8</sup> in the same year, fed two sets of dogs respectively, white of egg and calves' brains, and found no increase in the bile with the diet richer in cholesterol.

In 1908, Kusumoto<sup>9</sup> fed dogs with ordinary diet with and without the addition of cholesterol, and estimated the cholesterol of the feces, finding that an average of 30 per cent of the amount ingested failed of excretion through the intestines.

In the same year Dorée and Gardner,<sup>10</sup> in a convincing series of experiments, supported the above cited evidence favoring absorption of cholesterol from the food. In the feces of rabbits fed in the course of several days with 2 grams of cholesterol, preceded and followed by three days of feeding with a cholesterol-free diet, they found that at least 25 per cent, and often more, of the ingested cholesterol failed to appear in the feces. A rabbit receiving only the cholesterol-free diet excreted no cholesterol. In blood-estimations they employed the gravimetric method. Rabbits fed twenty days with cholesterol-free diet showed only a trace of the substance in the blood, whereas 0.0415 per cent cholesterol appeared in the blood of rabbits fed for ten days on the same diet with the addition of a total of 2.25 grams of cholesterol. With dogs they found some increase in the blood with foods rich in cholesterol. Fraser and Gardner<sup>11</sup> reported inhibition of saponin haemolysis by the serum of rabbits fed (1) with cholesterol, (2) with the cholesterol esters, (3) with mixed diet, as compared in each case with the serum of rabbits fed on cholesterol-free bran. The same authors,<sup>12</sup> a year later, employing a modification of the new Windaus digitonin method of cholesterol estima-

<sup>7</sup> Morgenroth and Reicher: *Berl. klin. Wochenschr.*, xxxviii, p. 1200, 1907.

<sup>8</sup> Goodman: *Hofmeister's Beiträge*, ix, p. 91, 1907; cited from Dorée and Gardner: *loc. cit.*

<sup>9</sup> Kusumoto: *Biochem. Zeitschr.*, xiv, p. 411, 1908.

<sup>10</sup> Dorée and Gardner: *Proc. of the Royal Society*, lxxxi, p. 109, 1908.

<sup>11</sup> Fraser and Gardner: *Proc. of the Royal Society*, lxxxi, p. 230, 1909.

<sup>12</sup> *Ibid.*, lxxxii, p. 559, 1910.



tion, repeated the work of Dorée and Gardner and reported similar results. Studies carried out in 1912 by Ellis and Gardner,<sup>13</sup> in the same laboratory, led them to conclude from evidence of the same nature, that the cholesterol content of the blood is dependent on the "sterol-content"—i. e., phytosterol and cholesterol—of the diet. An increase in the blood during starvation they attributed to the freeing of cholesterol by the destruction of the cholesterol-rich tissues of the animal.

Klein,<sup>14</sup> two years earlier, as reported by Magnus-Levy, found increase of absorption with increase of dose of cholesterol, as measured by the output in the feces of dogs. He found no difference in absorption between pure cholesterol and its esters.

Grigaut and L'Huillier,<sup>15</sup> in 1912, studied, with the former's colorimetric method, the curve of cholesterol content in the blood of dogs fed daily with 1 or 2 grams of cholesterol; and compared this curve with the curve of cholesterol present in the feces during the period of experimentation. His curves show a marked rise with the first feeding of cholesterol and a maintenance of the "hypercholestérinémie" throughout the feeding period, with a prompt fall coincident with the return to normal diet.

Anitschkow<sup>16</sup> found, after prolonged feeding of cholesterol, pathological changes in various tissues of rabbits, particularly the walls of the aorta, representing an increased body-content of cholesterol. Others report similar observations.

Rouzaud and Cabanis,<sup>17</sup> using the Grigaut method, found an increase in the blood of only one out of eleven healthy young people, four to five hours after the ingestion of a meal consisting of thin soup, bread, meat, green peas, two eggs and wine. The other ten subjects showed no change. Their work seems not to have been satisfactorily controlled.

For the purpose of this research, the sole conclusion to be drawn from these investigations is that, after the feeding of cholesterol and its esters in relatively large doses, there is an undoubted increase in the blood as compared to the average figures with any one method of estimation. They give no hint as to the rapidity of absorption into the blood or disappearance from it.

#### METHOD.

For the present investigation, rabbits were fed alike on the ordinary mixed diet of oats, hay or grass, and bread, and received daily as much as they would eat. They were thus under approximately normal conditions of metabolism.

<sup>13</sup> Ellis and Gardner: *Proc. of the Royal Society*, lxxxv, p. 385, 1912.

<sup>14</sup> Klein (Magnus-Levy): *Biochem. Zeitschr.*, xxix, p. 465, 1910.

<sup>15</sup> Grigaut and L'Huillier: *Compt. rend. soc. biol.*, lxxiii, p. 304, 1912.

<sup>16</sup> Anitschkow: *Deutsch. med. Wochenschr.*, 1913, p. 741.

<sup>17</sup> Rouzaud and Cabanis: *Compt. rend. soc. biol.*, lxxiv, p. 469, 1913.

About 6 cc. of blood were withdrawn for estimation and immediately thereafter 10 cc. of a 3 per cent solution of cholesterol in olive oil (Merck), representing a dose of 0.3 gram of cholesterol, was introduced into the stomach by a tube. The control animals, with the exception of one (XVII), received the same amount of a solution of pure olive oil.

The blood for the further estimations was withdrawn at intervals indicated in the tables to follow, in each instance about 6 cc. being taken. The degree of the consequent anemia was observed by haemoglobin determinations after Haldane. All bleeding was done by the Zahn method<sup>18</sup> with a suction-glass from the veins of the ear; and the blood was treated with sodium oxalate to prevent coagulation.

The Autenrieth-Funk<sup>19</sup> colorimetric method of cholesterol estimation, with chloroform extraction, was employed, and, owing to the small amount of blood required by this method, it was possible, in most instances, to make two independent extractions and determinations with each sample of blood. The results of the two determinations, in most cases, agreed closely, as the tables indicate. On account of the small percentage of cholesterol that the bloods yielded, it was found necessary to modify the technique, as the authors suggest in the original description, to the extent of extracting with 55 cc. instead of 100 cc., and, after the ordinary incidental evaporation, making up the test solution to 50 cc. Repeated further extractions with no addition to the yield of cholesterol showed that, with small percentages at least, the less extended extraction is as effective as a more thorough procedure.

#### RESULTS OF EXPERIMENTS.

The figures for the cholesterol content from the blood of twenty normal rabbits, as given in the seventeen tables below, together with those from three rabbits not there listed, range from a maximum of 0.1230 to a minimum of 0.0795 per cent with an average of 0.1020 per cent. This average is distinctly higher than that which Abderhalden<sup>20</sup> reported in 1898, determined by the gravimetric method from the mixed blood of twelve healthy rabbits. His figure for the whole blood is 0.0611 per cent.

<sup>18</sup> Zahn: *Münch. med. Wochenschr.*, 1912, No. 16, p. 861.

<sup>19</sup> Autenrieth and Funk: *Münch. med. Wochenschr.*, 1913, No. 23, p. 1243.

<sup>20</sup> Abderhalden: *Zeitschr. f. physiol. Chem.*, xxv, p. 65, 1898.

The records of the seventeen experiments and controls follow:

*Experimental series. Fed with cholesterol.*

*Rabbit I.* 1550 grams. 10 cc. 3 per cent cholesterol oil by mouth.

INTERVAL AFTER ADMINISTERING CHOLESTEROL	CHOLESTEROL IN BLOOD					
	Hgb.	Absolute Percentage			Increase	Decrease
		a	b	Average		
	per cent				per cent	per cent
Normal.....	74	0.1200	0.1195	0.1198		
6 hours.....		0.1185	0.1195	0.1190		0.67
3 days.....	60	0.1515	0.1490	0.1503	25.46	
19 days.....	73	0.1090	0.1050	0.1070		10.68

*Rabbit II.* 1650 grams. 10 cc. 3 per cent cholesterol oil by mouth.

Normal.....	60	0.1230		0.1230		
24 hours.....		0.1550	0.1580	0.1565	27.23	
5 days.....		0.1515	0.1535	0.1525	23.99	
19 days.....	60	0.1400		0.1400	13.82	

*Rabbit III.* 1650 grams. 10 cc. 3 per cent cholesterol oil by mouth.

Normal.....	75	0.1010	0.1020	0.1015		
6 hours.....		0.1050	0.1040	0.1045	2.95	
3 days.....	68	0.1074	0.1050	0.1062	4.63	
5 days.....	60	0.0950	0.0945	0.0948		6.60

*Rabbit IV.* 1520 grams. 10 cc. 3 per cent cholesterol oil by mouth.

Normal.....	80	0.0920	0.0920	0.0920		
24 hours.....		0.1190	0.1175	0.1183	28.58	
4 days.....	76	0.1050		0.1050	14.13	
6 days.....	70	0.1065	0.1050	0.1058	15.00	
18 days.....	78	0.1015	0.1040	0.1028	11.74	

*Rabbit V.* 2340 grams. 10 cc. 3 per cent cholesterol oil by mouth.

Normal.....	75	0.0900	0.0860	0.0880		
8 hours.....		0.0960	0.0965	0.0963	9.43	
2 days.....		0.0965	0.0940	0.0953	8.29	

*Rabbit VI.* 2570 grams. 10 cc. 3 per cent cholesterol oil by mouth.

Normal.....	70	0.1120		0.1120		
12 hours.....		0.1190	0.1165	0.1178	5.18	
3 days.....		0.1170	0.1050	0.1110		0.89

*Experimental series. Fed with chole.*

*Rabbit VII. 1930 grams. 10 cc. 3 per cent*

INTERVAL AFTER ADMINISTERING CHOLESTEROL	Hgb.	CHOLESTEROL	
		Absolute Percent	
		a	b
		<i>per cent</i>	
Normal.....	70	0.1090	0.1105
12 hours.....		0.1190	0.1220
3 days.....		0.1685	0.1715
6 days.....	64	0.1720	0.1780

*Rabbit VIII. 1700 grams. 10 cc. 3 per cent*

Normal.....	76	0.1070	0.1060
24 hours.....		0.1150	0.1170
3 days.....	68	0.1055	0.1000

*Rabbit IX. 1750 grams. 10 cc. 3 per cent*

Normal.....	92	0.0900	0.0910
6 hours.....		0.0795	0.0820
2 days.....	75	0.1130	

*Rabbit X. 1700 grams. 10 cc. 3 per cent ch*

Normal.....	96	0.0810	0.0820
6 hours.....		0.0730	0.0825
3 days.....	82	0.0955	0.0950

*Rabbit XI. 1610 grams. 10 cc. 3 per cent c*

Normal.....	74	0.0910	0.0910
8 hours.....		0.0790	0.0900

*Rabbit XII. 1570 grams. 10 cc. 3 per cent c*

Normal.....	70	0.0855	0.0880
8 hours.....		0.0680	

*Control series.**Rabbit XIII.* 1450 grams. Control. 10 cc. olive oil by mouth.

INTERVAL AFTER ADMINISTERING OLIVE OIL	CHOLESTEROL IN BLOOD					
	Hgb.	Absolute Percentage			Increase	Decrease
		a	b	Average		
	per cent				per cent	per cent
Normal.....	78	0.0865	0.0870	0.0868		
6 hours.....		0.1100	0.1095	0.1098	26.49	
3 days.....	60	0.0972		0.0972	11.98	

*Rabbit XIV.* 1480 grams. Control. 10 cc. olive oil by mouth.

Normal.....	82	0.1100	0.1095	0.1098		
6 hours.....		0.1185	0.1170	0.1178	7.29	
3 days.....	68	0.1110	0.1140	0.1125	2.46	
5 days.....	62	0.1150		0.1150	4.73	

*Rabbit XV.* 1700 grams. Control. 10 cc. olive oil by mouth.

Normal.....	72	0.0770	0.0820	0.0795		
12 hours.....		0.0760	0.0780	0.0770		3.14
3 days.....		0.0815	0.0790	0.0803	1.00	

*Rabbit XVI.* 1900 grams. Control. 10 cc. olive oil by mouth.

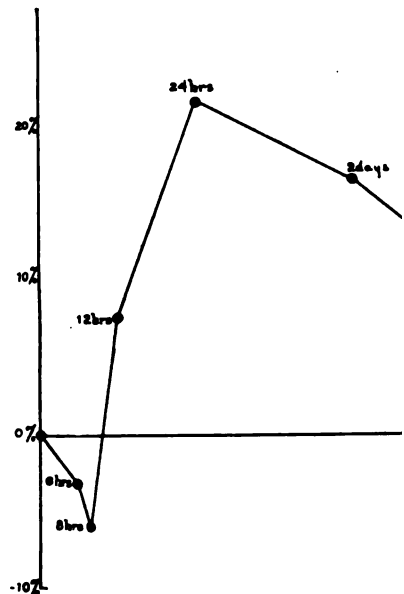
Normal.....	70	0.0990	0.1020	0.1005		
24 hours.....		0.1015	0.1045	0.1030	2.48	
4 days.....	70	0.1000	0.0955	0.0978		2.68

*Rabbit XVII.* 1920 grams. Control. Received nothing.

Normal.....	76	0.1130	0.1145	0.1138		
6 hours.....		0.1070	0.1095	0.1083		4.83

From the above figures the appended curve was constructed, showing the average blood-content at the intervals indicated in the twelve animals that received the usual dose of cholesterol. It must be noted, that, although the points form a not irregular line, the average figure for any one interval is, in most cases, drawn from widely differing individual figures. Thus at six hours, the average indicated on the curve, -3.24 per cent, is a product of results varying from -10.72 per cent to +2.95 per cent. In reckoning the

average figure for three days, it was necessary to include Rabbit VII, which shows at that interval a value as great as the next greatest in the series and as great as the average; this discrepancy is not to be accounted for. X shows the deflection of the curve if the blood from Rabbit VII is allowed to return to the average.



Composite curve from seventeen rabbits, showing the change in cholesterol content of the blood after the feeding of cholesterol.

The great differences in the individual experimental animals and in the controls, are dependent on the body weight. Although the heaviest in the series, are among those that follow from the procedure, yet Rabbit III, although the heaviest, shows even less effect, and Rabbit VII, although the lightest, shows the greatest effect of withdrawal of blood is alone sufficient to bring the cholesterol content from the normal level to the level suggested by the curve.

<sup>21</sup> Mauriac: *Compt. rend. soc. biol.*, lxxiii, p. 100.

possible, but an analysis of the figures shows that the control animals show less average variation from the normal than the experimental animals. There is one notable exception; a single control, Rabbit XIII, fed with pure olive oil, at the end of six hours showed an increase of 26.49 per cent whereas among the animals fed with cholesterol it will be noted that in only one instance (III) was there an increase so soon after feeding, and in that case an increase of only 2.95 per cent. The reason for this exceptional occurrence in Rabbit XIII is unexplained. This, however, can hardly vitiate the conclusion that, in general, the curve established from the estimations on the blood of the cholesterol-fed animals is based on actual absorption of the cholesterol placed in the digestive tract and not on properties of the technique employed.

To turn to the figures from the cholesterol-fed animals from which the curve was constructed, we find that several of the animals, as mentioned above (III, V, VI), show less, or little more, increase of cholesterol in the blood after the administration of cholesterol than do the animals that received no cholesterol. The majority however, tend to correspond with the curve—to show an initial fall (due, perhaps, to the recent hæmorrhage with dilution of the diminished blood-volume from tissue-fluids) lasting for six to eight hours, followed by a rise reaching its maximum at the end of about twenty-four hours, and a more gradual fall through the next two or more days.

The small number of experiments performed does not permit more extended generalization; one must expect that any individual rabbit may depart widely from the tendency that the curve expresses.<sup>22</sup>

#### CONCLUSIONS.

It is possible by giving rabbits small doses of cholesterol by mouth to demonstrate in the majority of instances, an increase of this substance in the blood in the course of a few hours.

<sup>22</sup> Three experiments, with subcutaneous injection of cholesterol oil in 10 cc. amounts suggest that the absorption from the subcutaneous tissues is much slower. The maximum amount in the blood appeared to be reached between the third and sixth days or even later. These animals were not controlled and the other conditions of the experiments make the results worth recording only as being suggestive.





## GLYOXALASE. PART IV.

By H. D. DAKIN AND H. W. DUDLEY.

(From the Herter Laboratory, New York.)

(Received for publication, November 27, 1913.)

The object of the following paper is to record some new experiments dealing with enzymes of the glyoxalase type and with the inhibitory action of the pancreas upon these enzymes. It may be recalled that we have recorded the presence of glyoxalases in a variety of tissues from various animal species.<sup>1</sup> The tissues examined with positive results included liver, kidney, thyroid, spleen, heart muscle, skeletal muscle, tongue, lung, brain, blood cells and gastric mucosa. Negative results were obtained with saliva, urine, bile and blood serum. On the other hand, pancreatic tissue and juice were found not only to be free from glyoxalase but to contain a thermolabile substance, not improbably an enzyme, which exerts an intense inhibitory action upon glyoxalase derived from other sources. The inhibitory substance, named for convenience antiglyoxalase, is not identical with trypsin, lipase or diastase.

The inhibitory action of the pancreas upon glyoxalase appeared so definite a phenomenon and had so suggestive a relation to the function of the pancreas in carbohydrate and lactic acid metabolism, that we considered it desirable to examine other glands of the body, particularly those which in recent years have been brought into relation with sugar metabolism, in order to learn if antiglyoxalase was peculiar to the pancreas.

We have been unable to obtain any evidence of the presence of antiglyoxalase in the thymus, thyroid, suprarenal, pituitary, or salivary glands, or testicle, but on the contrary we have determined the presence of glyoxalase in all of these organs. The amount of glyoxalase in the salivary glands is very small. In the case of the abdominal lymph glands of the dog, we have observed a con-

<sup>1</sup> This *Journal*, xv, p. 463, 1913.

stant absence of glyoxalase, but efforts to obtain evidence of the presence of antiglyoxalase have given doubtful or negative results. It is certain, however, that if antiglyoxalase be present in the lymph glands, its amount is utterly insignificant when compared with the pancreas. It is perhaps conceivable that the antiglyoxalase of the pancreas reaches distant parts of the body by way of the lymphatic system, but of this we have no precise evidence. Moreover, we are inclined to believe that, notwithstanding the fact that antiglyoxalase is present in the external pancreatic secretion, it is likely that antiglyoxalase is largely contained in an internal secretion. The reasons for this belief are as follows: In the first place we are under the impression that the concentration of antiglyoxalase in pancreatic juice is relatively small compared with that in the tissues. Secondly, antiglyoxalase appears to be non-dialyzable and does not pass through animal membranes and hence would probably not undergo ready absorption from the intestine. In the light of our present results, it appears that the production of antiglyoxalase is a specific function of the pancreas.

We wish to record at this point that in a private communication from Prof. F. G. Hopkins we learn that in some experiments made several years ago, he found that lactic acid production in muscle was markedly inhibited by the action of pancreas extract. We understand that these experiments are being amplified and will be published shortly.

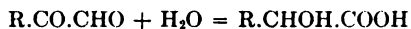
In a recent paper Neuberg has taken exception to the name glyoxalase. He writes:<sup>2</sup>

<sup>1</sup> Das Enzym, das die Umwandlung von Methylglyoxal in Milchsäure bewirkt, ähnelt nach meinen Ausführungen in seinen Eigenschaften und seiner Wirkungsweise der bekannten Aldehydmutase. Durchgreifende Unterschiede von dieser sind nicht offenbar geworden. Es liegt daher bislang keine Veranlassung vor, das Ferment als Vertreter einer neuen Gruppe zu betrachten, wie es Dakin tut. Besonders ist aber der von Dakin gewählte Name "Glyoxalase" höchst unglücklich, da gerade das Glyoxal bisher nicht nachweisbar beeinflusst wird. Zweckmässiger erscheint daher mein Vorschlag (l. c.), das Enzym den Aldehydasen anzureihen und es vorläufig Ketonaldehydmutase zu benennen, da diese Bezeichnung nichts präjudiziert.

<sup>2</sup> *Biochem. Zeitschr.*, lv, p. 502, 1913.

We believe that these objections are without weight for the following reasons. In the first place, contrary to Neuberg, we find that glyoxal is converted into glycollic acid by enzyme action. On perfusing a dog's liver with blood to which glyoxal had been added, we recovered almost 2 grams of pure calcium glycollate. Since ordinary commercial glyoxal is a mixture of highly polymerized substances, it is not surprising that it should be acted on less readily than some of the other glyoxals.

Secondly, we have found that the inhibitory action of pancreas extract upon glyoxalase furnishes us with an excellent method for the differentiation of glyoxalase from other enzymes. Parnas' aldehydemutase is scarcely affected by pancreas extract under conditions which completely inhibit glyoxalase, so that we believe that the two enzymes can have nothing in common. Incidentally it may be noted that the distribution of the two enzymes in the body is quite different and the reactions with which they severally are concerned have only a superficial resemblance. We therefore propose to retain the name "glyoxalase" for the enzymes which we have shown to effect the conversion of various glyoxals into the corresponding hydroxy-acids:



Thus far we have made use of glyoxal itself, methyl glyoxal, isobutyl glyoxal, phenyl glyoxal and benzyl glyoxal, and in every case we have obtained the corresponding hydroxy-acid by the action of glyoxalase. Finally we wish to mention that we have been able to demonstrate the formation of amino- as well as hydroxy-acids from corresponding glyoxals when perfused through the liver. We are also making experiments in which the formation of hydroxy-acid is suppressed by addition of pancreas extract to the blood used for perfusion. A description of the synthesis of the hitherto unknown isobutyl and benzyl glyoxals, corresponding to leucine and phenylalanine, together with a study of the formation of amino- and hydroxy-acids from them, will be published shortly.

## EXPERIMENTAL.

*I. Glyoxalase in certain*

The method employed was identical with that described in a previous paper,<sup>3</sup> except that instead of using extracts the tissues themselves from blood were added to the digestion mixture. Mandelic acid was used as substrate and in all positive cases the acid produced was isolated in crystalline form.

From blank experiments in which the tissues were not heated preliminary heating, no mandelic acid was produced. The following results are typical.

ANIMAL	TISSUE	
Calf.....	Thymus	(10 gms.)
Horse.....	Suprarenal	(20 gms.)
	Pituitary	( 3 gms.)
Ox.....	Pituitary	(10 gms.)
	Testicle	(10 gms.)
Dog.....	Abdom. lymph glands	( 5 gms.)
	Abdom. lymph glands	( 6 gms.)
	Salivary glands	(10 gms.)

We owe the experiments on the suprarenal of the horse to the kindness of Dr. C. C. Armstrong and we wish to express our thanks also to the research laboratories of Armour and Company for experiments with ox pituitary.

*II. Examination of abdominal lymph glands for antglyoxalase.*

Many experiments were made in which abdominal lymph glands<sup>4</sup> were added to 20 per cent skeletal

<sup>3</sup> This *Journal*, xv, p. 466, 1913.

<sup>4</sup> Glands from near the pancreas were purpo

incubating this mixture for several hours in the presence of chalk, phenyl glyoxal was added and incubation continued for about eighteen hours. As control an equal amount of 20 per cent extract was incubated in the presence of chalk, phenyl glyoxal being added at the same time as to the lymph gland experiment. Only a few typical experiments are here reported.

a. 50 cc. 20 per cent skeletal muscle extract were incubated for three hours with 9 gms. minced lymph glands and 5 cc. of a chalk suspension. Then 0.2 gm. phenyl glyoxal was added and incubation continued for twenty hours.

In the control experiment 50 cc. of the same muscle extract were incubated in the presence of chalk for three hours, after which 0.2 gm. phenyl glyoxal was added and incubation continued for twenty hours.

Lymph gland experiment. Rotation of mandelic acid:  $-0.82^{\circ}$ ; Acidity, 3.8 cc.

Control. Rotation of mandelic acid:  $-1.35^{\circ}$ ; Acidity, 3.8 cc.

b. As in (a), 14 gms. lymph glands being used.

Lymph gland experiment. Rotation of mandelic acid:  $-0.37^{\circ}$ ; Acidity, 3.2 cc.

Control. Rotation of mandelic acid:  $-0.22^{\circ}$ ; Acidity, 2.0 cc.

c. As in (a) 5 gms. lymph glands taken.

Lymph gland experiment. Rotation of mandelic acid:  $0^{\circ}$ ; Acidity, 1.8 cc.

Control. Rotation of mandelic acid:  $-0.32^{\circ}$ ; Acidity, 1.8 cc.

d. As in (a) 3.5 gms. lymph glands added.

Lymph gland experiment. Rotation of mandelic acid:  $-0.18^{\circ}$ ; Acidity, 2.2 cc.

Control. Rotation of mandelic acid:  $-0.17^{\circ}$ ; Acidity, 2.2 cc.

The results of these experiments show that under the observed conditions the presence of antiglyoxalase in lymph glands cannot be definitely asserted.

### III. Dialysis experiments with antiglyoxalase.

Ten grams of fresh dog's pancreas were ground up with sand mixed with 50 cc. of water and dialyzed for twenty hours in a condome made from the caecum of the sheep. This was chosen as an appropriate membrane to employ in this experiment. The contents of the condome and the dialysate were then tested for antiglyoxalase by adding them each to 50 cc. of 20 per cent dog's skeletal muscle extract and incubating for three hours before adding phenyl glyoxal, at the same time carrying out a control.

experiment with the muscle extract. The following results were obtained:

Muscle extract. Rotation of mandelic acid:  $-0.9^{\circ}$ ; Acidity, 4.4. cc.

Muscle extract + contents of dialyzer. Rotation of mandelic acid:  $+0.1^{\circ}$ ; Acidity, 0.5 cc.

Muscle extract + dialysate. Rotation of mandelic acid:  $-0.85^{\circ}$ ; Acidity, 4.6 cc.

It will be noted that there is no inhibition in the third experiment, whereas in the second the enzyme has been completely paralyzed showing that no antiglyoxalase has passed through the membrane.

#### *IV. Formation of glycollic acid from glyoxal in the liver.*

The technique of perfusion was similar to that already reported<sup>5</sup> in connection with other experiments made in this laboratory. The dog (4.8 kgm.) was starved for twenty-four hours previous to the operation. The liver (170 grams) was perfused with a mixture containing 170 cc. of the animal's own blood, 500 cc. of fresh blood from another dog and 200 cc. of saline. During the first ten minutes of the perfusion solutions of about 5 grams glyoxal in 100 cc. water and of 5 grams sodium bicarbonate in 200 cc. water were added in small portions to the perfusion fluid. At the end of the perfusion, which lasted an hour, the liver was washed out with 200 cc. of saline. The perfusion fluid, after removal of proteins according to Schenck's method, when tested with *p*-nitrophenylhydrazine, gave a precipitate of the characteristic dinitrophenylhydrazone of glyoxal, showing that there was still unchanged glyoxal present.

An acetone determination of an aliquot part of the filtrate showed that only 29 mgm. of acetoacetic acid had been formed.

The mercury was removed from the clear filtrate of the perfusion fluid by means of hydrogen sulphide and the still acid liquid was then evaporated to dryness *in vacuo*. The residue was washed with alcohol, the alcohol extract evaporated, taken up in water and after the addition of ammonium sulphate and phosphoric acid, extracted with ether in a continuous extractor. The ether

<sup>5</sup> This *Journal*, ix, p. 146, 1911.

extract, after treatment with calcium carbonate gave a salt crystallizing in the characteristic form of calcium glycolate.

Almost 2 grams of crystallized calcium glycolate were obtained.

Analysis of air-dried salt: 0.1176 gm. lost 0.0260 gm.  $H_2O$  at  $140^\circ$  and gave 0.0269 gm.  $CaO$ .

	Found:	Calculated for $CaC_4H_7O_6 \cdot 3H_2O$ :
$H_2O$ .....	22.1	22.1 per cent.
Ca.....	16.4	16.4 per cent.

The remainder of the calcium salt was decomposed with oxalic acid, the filtrate evaporated and taken up in ether. The residue from the clear ether solution crystallized at once on seeding with a crystal of glycollic acid, and the crystals so obtained, dried on porous plate, melted at  $76-78^\circ$ .

*V. The differentiation of glyoxalase from aldehydemutase by means of the action of pancreas extract.*

As is known, an extract of pancreas inhibits the action of glyoxalase. The following experiment was made to determine whether pancreatic extract exerted a similar effect on aldehydemutase, an enzyme described by Battelli and Stern<sup>6</sup> and also by Parnas,<sup>7</sup> whose method of investigation was substantially followed.

The enzyme extract was made by stirring water with an equal weight of minced ox liver and straining through muslin. 250 cc. of this extract were then placed in each of four flasks. To the contents of flasks (1) and (2) were added 2.1 grams of sodium bicarbonate and 2 cc. of isovaleric aldehyde. The extract in flask (3) was digested forty minutes with 1 gram pancreatin before adding 2.1 grams sodium bicarbonate and 2 cc. isovaleric aldehyde. Flask (4) was heated on the water bath for fifteen minutes and then similar amounts of sodium bicarbonate and isovaleric aldehyde were added.

All the mixtures were incubated at  $37^\circ$  for five and one-half hours after addition of the aldehyde. The reaction was then checked by adding 10 cc. of phosphoric acid, and the mixtures were immediately distilled in steam, 500 cc. of distillate being collected

<sup>6</sup> *Biochem. Zeitschr.*, xxviii, p. 147, 1910; xxix, p. 130, 1910.

<sup>7</sup> *Ibid.*, xxviii, p. 274, 1910.

in each case. The distillates were then titrated, the results being as follows:

- (1) Acidity = 6.7 cc.
- (2) Acidity = 6.4 cc.
- (3) Acidity = 5.7 cc. Pancreas added.
- (4) Acidity = 2.7 cc. Blank experiment.

This experiment shows clearly that the action of the enzyme aldehydemutase is not abolished by pancreas extract, as is the case with glyoxalase.

A second experiment was made in order to compare directly the aldehydemutase and glyoxalase contained in the same extract both before and after treatment with pancreas extract. To this end an emulsion of dog's liver in five times its weight of water was taken. In two flasks (1) and (2) were placed 400 cc. of the emulsion and to flask (2) was added the dog's pancreas (16 grams) finely minced. In flask (3) 350 cc. of the liver emulsion were heated up on the water bath for fifteen minutes to act as a control in the aldehydemutase determinations.

After flasks (1) and (2) had been incubated at 37° for two hours, 50 cc. from each were measured off into flasks (4) and (5) respectively; to these were added 0.2 gram phenyl glyoxal and 5 cc. of a chalk suspension and a typical glyoxalase determination was carried out.

To each of flasks (1), (2) and (3) were added 3 grams of sodium bicarbonate and 3 cc. of isovaleric aldehyde. Incubation was continued for fifteen hours, when the reaction was checked by the addition of 15 cc. of phosphoric acid and the mixtures were immediately distilled in steam, 400 cc. being collected in each distillation. The distillates were titrated, giving figures indicative of the activity of aldehydemutase, while the determination of the mandelic acid produced in flasks (4) and (5) is a measure of the glyoxalase contents of the same mixtures.

- Aldehydemutase:* Flask 1. Pancreas absent. Acidity = 3.2 cc.  
 " Flask 2. Pancreas present. Acidity = 3.0 cc.  
 Flask 3. Blank. Acidity = 1.6 cc.
- Glyoxalase:* Flask 4. Pancreas absent. Rotation of mandelic acid,  
 -1.13°; Acidity, 2.0 cc.  
 Flask 5. Pancreas present. Rotation of mandelic acid,  
 -0.12°; Acidity, 0.25 cc.



The glyoxalase determinations (4) and (5) were actually made on samples taken from flasks (1) and (2).

It is seen that the activity of aldehydemutase is not appreciably diminished by the action of pancreas extract, while the glyoxalase under the same treatment is practically completely inhibited. The two enzymes are undoubtedly distinct.

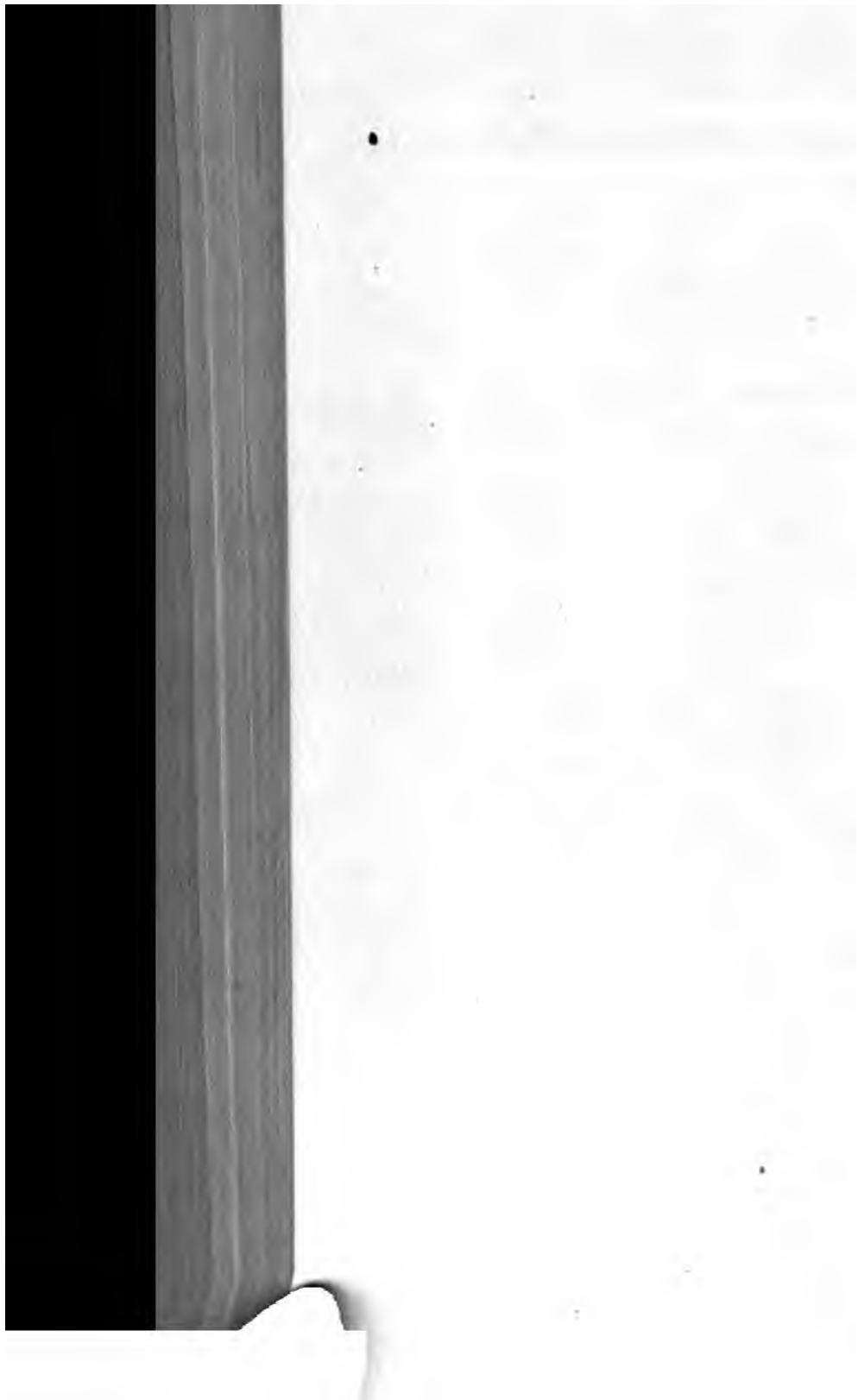
#### SUMMARY.

The presence of glyoxalase and the absence of antiglyoxalase has been determined in all the glands of the body we have examined, with the exception of the pancreas and abdominal lymphatic glands.

The lymphatic glands contain no glyoxalase and compared with the pancreas, the inhibitory action of extracts of lymph glands upon glyoxalase is trifling or non-existent.

The formation of antiglyoxalase, so far as can be at present determined, appears to be a specific function of the pancreas, and some reasons are adduced for suspecting that it acts mainly by way of an internal secretion.

Contrary to Neuberg's statement, we find that glyoxal may be converted into glycollic acid by enzyme action. Furthermore we find that Neuberg's suggested relation between glyoxalase and aldehydemutase is incorrect. We have shown that the enzymes are entirely distinct since, unlike glyoxalase, aldehydemutase is substantially unaffected by pancreas extract.



## SOME NEGATIVE EXPERIMENTS ON THE INFLUENCE OF THE PANCREAS UPON ACETOACETIC ACID FORMATION IN THE LIVER.

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(*From the Herter Laboratory, New York.*)

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The incentive to make the following experiments to determine a possible influence of the pancreas upon acetoacetic acid formation in the liver arose from the following facts. First, the known acidosis with excretion of acetoacetic acid observed to follow extirpation of the pancreas. Second, the fact that we have found in the pancreas a mechanism for the regulation of the action of the enzyme glyoxalase, whose function, at least in part, is concerned with the formation of another acid produced in intermediary metabolism, namely, lactic acid. Third, the fact that it is much more difficult to evoke a considerable excretion of acetoacetic acid in the non-diabetic intact animal under normal conditions than it is to demonstrate its production in the excised liver on perfusion.

It seemed possible that the pancreas might furnish some enzyme or hormone the absence of which leads to acidosis in the diabetic animal. So far as we are aware, no experiments have hitherto been made to determine this point, although a number of workers have investigated the influence of the pancreas upon the capacity of various tissues to effect the oxidation of glucose. More recently Paderi<sup>1</sup> has found that the addition of pancreas extract to the fluid used for perfusing a glycogen-containing liver, was not followed by a diminished glucose production.

In our attempt to detect any influence that the pancreas may have on acetoacetic acid production we have perfused dogs' livers with blood containing added substances, known from Embden's

<sup>1</sup> *Arch. d. pharmacol. experim.*, xvi, p. 54, 1913.

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experiments to yield acetoacetic acid from sodium salts of butyric and homogentisic acid were chosen. In some of the experiments whole pancreas to the blood used for perfusion, skeletal muscle extract or heated pancreas control. The tissue extracts were prepared from perfectly fresh tissue with sand and ten parts of water. A suspension was then strained, a suitable amount of the suspension was then whipped with clotting blood and added to the blood used for perfusion. The methods of analysis were those previously used.

Although the results of our experiments may be considered to show a slightly lessened formation of acetoacetic acid in those experiments in which pancreas extract was added to the blood, we believe that the results do not warrant a different interpretation, owing to wide individual differences in the results of the experiments.

We conclude that under the condition of pancreas extract to the blood the formation of acetoacetic acid in the liver from butyric acid or tyrosine.

SUBSTANCE ADDED	TISSUE EXTRACT AND BLOOD
1. Butyric acid 2 gms.....	100 cc. pancrea
2. Butyric acid 2 gms.....	250 cc. pancrea
3. Butyric acid 2 gms.....	100 cc. heated
4. Butyric acid 2 gms.....	100 cc. muscle
5. Tyrosine 1 gm.....	100 cc. pancrea
6. Tyrosine 2 gms.....	200 cc. pancrea
7. Tyrosine 2 gms.....	200 cc. pancrea
8. Tyrosine 2 gms.....	200 cc. heated
9. Homogentisic acid 2 gms.	100 cc. pancrea
10. Homogentisic acid 2 gms.	200 cc. pancrea
11. Homogentisic acid 2 gms.	100 cc. muscle

## ON FAT ABSORPTION.

### III. CHANGES IN FAT DURING ABSORPTION.

By W. R. BLOOR.

(From the Laboratories of Biological Chemistry of Washington University,  
St. Louis, Mo.)

(Received for publication, November 28, 1913.)

The belief regarding fat absorption which is now almost universally accepted is that the fats are saponified in the intestine, absorbed in water-soluble form as soaps, and resynthesized into neutral fats during the passage through the absorbing cells. The more important evidence<sup>1</sup> in support of this belief is as follows:

1. Fatty substances such as glycerides, other readily saponifiable esters, fatty acids and soaps, lecithin, etc., which are soluble in water or can be changed by digestion into compounds soluble in water (or bile) at body temperature are readily absorbed. They appear in the chyle as triglycerides.

2. Fatty substances which cannot be changed into water-soluble form in the intestine under these conditions are not absorbed, *no matter in what form they are presented*. In this class fall difficultly saponifiable esters such as those of wool fat, also the petroleum hydrocarbons, etc.,—substances which are soluble in the ordinary fats and fat solvents, and in most cases form very good emulsions with water.

3. The abundant provision made for saponification and for the absorption of soaps in the intestine by the supply of large amounts of lipase and bile—whose chief function is now believed to be to aid in fat absorption.

4. The presence of soaps in the intestine in relatively large proportion, while only small amounts are present in the chyle.

5. While emulsified neutral fats are found on both sides of the absorbing cells, the particles of emulsified fat in the lacteals are

<sup>1</sup> The evidence is discussed in greater detail in the preceding paper of this series: this *Journal*, xv, p. 105, 1913.

very much finer than those in the intestine. They are of "dust-like fineness,"<sup>2</sup> apparently of the same order of magnitude as the "haemakonien" of Neisser,<sup>3</sup> and are probably formed by the partial flocking of the newly resynthesized fat molecules under the influence of the electrolytes of the lymph stream.

The purpose of the complete breaking down of the fats in the intestine and their immediate resynthesis in the passage from the intestine is not clear in the light of our present knowledge. One reason which has been suggested<sup>4</sup> is that the process is a protective one for the purpose of excluding undesirable fatty substances, such as wool-fat and the petroleum hydrocarbons, which differ from ordinary food fats mainly in that they cannot be changed into water-soluble substances in the intestine, and which but for this mechanism would be absorbed with the fats. But this reason is obviously not sufficient to explain the changes, since these substances rarely occur in the food. A comparison of the absorption of fats with that of proteins and carbohydrates suggests another reason. It is now believed that during digestion all (organic) food-stuffs alike are broken down into their component "Bausteine" in the intestine, the object being to provide material in a sufficiently elementary form for use in building up the characteristic body tissue. Proteins are broken down to amino-acids, carbohydrates to monosaccharides and fats to fatty acids and glycerins. Protein and carbohydrate "Bausteine" pass directly into the blood stream and are not rebuilt into body protein and carbohydrate complexes (accepting the findings of Folin and Denis) until they reach the tissues and organs. In their passage from the intestine to the system they pass through the liver. Fats are unique in that they are rebuilt *before they leave the intestinal wall*, and entering the blood stream by way of the thoracic duct, avoid the liver, which appears to take part in fat metabolism only after the fats have passed to the tissues. The protein complexes rebuilt by the tissues from the protein building stones are different from the proteins ingested and are characteristic of the species, and of the tissue. Since the fats are rebuilt during their passage through the intestinal wall, it is logical to expect a change in their chemi-

<sup>2</sup> Munk: *Virchow's Archiv*, cxxiii, p. 239, 1891.

<sup>3</sup> Neisser and Brätning: *Zeitschr. f. exp. Path. u. Ther.*, iv, p. 747, 1907.

<sup>4</sup> Bloor: *loc. cit.*

cal structure during resynthesis analogous to that undergone by the proteins in the tissues. Another, and perhaps the main reason, then, for the phenomena of fat absorption may be looked for in changes which the fats undergo during absorption. What changes may be expected in the fats during absorption? The chemical structure of the fats is not definitely known, but it is believed that many fats contain in addition to simple triglycerides a considerable proportion of mixed triglycerides. The presence of mixed triglycerides is significant because many lecithins and similar substances contain a mixed glyceride residue; and since these lipoids are more closely identified with tissue structure than the fats, it may be assumed that the mixed glycerides are of special importance in those phases of fat metabolism which have to do with tissue repair. That mixed glycerides are also potentially optically active may also be considered significant in view of the possible importance of molecular structure in the utilization of other foodstuffs.<sup>5</sup>

We may look for two kinds of change in the fats during their passage through the intestinal wall: (a) a physical change consisting in a rearrangement of the quantities of the different glycerides—more or less of the liquid fats or of the solid fats—resulting in a mixture of different physical properties which may be more suitable for transport or storage than the fat fed, or (b) a chemical change consisting in a rearrangement of the fatty acids *in the molecules* of some or all of the glycerides, which in addition to changing their physical properties, would make them presumably more adaptable for use as tissue fats—for “endogenous” metabolism.

#### *Observed changes in fats during absorption.*

That fats do not pass into the chyle in exactly the form in which they occur in the food has been noted by several observers. Arnschink<sup>6</sup> on feeding mutton fat to dogs found that the feces fat had a higher melting point than the food fat and drew the

<sup>5</sup> An excellent example of the influence of molecular arrangement on protein utilization is reported in the recent work of Dakin and Dudley (this *Journal*, xv, p. 271, 1913) on racemized casein.

<sup>6</sup> Arnschink: *Zeitschr. f. Biol.*, xxvi, p. 434, 1890.

conclusion that there was a discrimination in favor of the softer fats during absorption. Munk<sup>7</sup> experimenting with a case of human chyle fistula after feeding mutton tallow found that the chyle fat had a lower melting point than the food fat, thus supplementing the findings of Arnschink. He explained the change also as being due to a discrimination in favor of the softer fats. Some results which he obtained later with the same patient indicate, however, that there is another factor. After excluding other fat from the diet, and feeding his patient cetyl palmitate, m.p. 55°, he found that the chyle contained in addition to palmitic acid (as triglyceride) about 14 per cent of combined oleic acid, also that the chyle fat had a melting point of 36°C. while tripalmitin melts at 65°C. He proved that the oleic acid could not have come from the food and must therefore have been supplied by the intestine.

Frank<sup>8</sup> in feeding experiments with dogs using fats of different melting points found that the melting point of the chyle fat was generally closer to that of the body temperature of the animal than that of the fat fed. In the same paper (Experiment 11) after feeding ethyl palmitate, he found that the melting point of the chyle fatty acids was 50.5°C. (palmitic acid 63°), while their iodine number was 32.6—corresponding to an oleic acid content of 36 per cent. In Experiment 12, after feeding ethyl palmitate (other fats were excluded from the diet in both experiments) and fractionating the chyle fat, one fraction was obtained which melted at 39° and had an oleic acid content of 25 per cent<sup>9</sup> (calculated from the iodine number). As in Munk's experiment the oleic acid was demonstrated not to have come from the food and was believed to have been supplied by the intestine or liver (via the bile).

From calculations based on figures for the chyle fat (per hour) of dogs given by v. Walther<sup>10</sup> and by Munk,<sup>11</sup> Frank concluded

<sup>7</sup> Munk: *loc. cit.*

<sup>8</sup> Frank: *Zeitschr. f. Biol.*, xxxvi, p. 568, 1898.

<sup>9</sup> It is worthy of note that an oleo dipalmitin (oleic acid content = 33.8 per cent) prepared by Kreis and Hafner (*Zeitschr. f. Untersuch. d. Nahr. u. Genussm.*, 1904, p. 665) had a melting point of 37–39°. Its calculated iodine number would be 30.4.

<sup>10</sup> v. Walther: *DuBois Raymond's Archiv*, 1890, p. 329.

<sup>11</sup> Munk: *loc. cit.*



that the extra fat found mixed with the food fat in the chyle, was only that normally present in the chyle and was not a purposive addition. A fact which he overlooked, however, was that the dogs used by Munk and v. Walther were as a rule very much larger than his dogs and would therefore yield more chyle per hour. Recalculating his results on the more logical percentage basis, it will be seen that even if all the fat of the fasting chyle were considered as oleic acid, the fat of fasting dog chyle does not account for the amounts of oleic acid found in his experiments. For example in Experiment 11, p. 576, 27 cc. of chyle yielded 0.372 gram of fat containing 40 per cent oleic acid = 0.14 gram of oleic acid.

27 cc. of fasting dog chyle would have yielded 0.07 gram of fat (0.25 per cent of the chyle—average of Munk's and V. Walther's figures).

In Experiment 12, p. 576, 40 cc. of chyle contained 1.43 grams fat with 13 per cent oleic acid = 0.186 gram; 40 cc. of fasting dog chyle contains 0.1 gram of fat.

Since, as will be shown later, the fat of fasting dog chyle does not consist entirely of oleic acid, the fact of these additions to the chyle fat becomes the more remarkable.

Bloor,<sup>12</sup> after feeding pure isomannid dilaurate, found that the purified chyle fat had a melting point of 32° (pure trilaurin melts at 45–46°). The fatty acids prepared from the chyle fat had a melting point of 30°, a mean molecular weight of 211, and an iodine number of 16.5. The corresponding figures for lauric acid are: m.p., 43.6°; mean molecular weight, 200; iodine number, 0. Calculating the unsaturated fatty acids as oleic acid from the iodine number, 18 per cent of the chyle fatty acids was oleic acid. The mean molecular weight of the chyle fatty acids, assuming them to consist only of lauric and oleic acids would then have been 215.3. Since the actual mean molecular weight was 211, oleic acid was probably not the only acid added, although it was the main one. Taking into account in this experiment the amount of chyle collected and accepting the figures of Munk and v. Walther for the fat content of fasting dog chyle it cannot be denied that the added oleic acid may have had its origin in this experiment in fat normally present in the fasting chyle.

<sup>12</sup> This *Journal*, xi, p. 429, 1912.

Raper,<sup>13</sup> after feeding cocoanut oil, with a considerable amount of lower fatty acids, observed differences between the food fatty acids and Cocoanut oil fatty acids: mean molecular number, 7.7. Chyle fatty acids: mean molecular number, 19.1. The change in molecular number is probably greater than could be accounted for from which (and other evidence) Raper concluded that fatty acids were absorbed through some other mechanism.

Bloor,<sup>14</sup> in feeding experiments with coconut oil, found what similar differences between the food fat and the fat of the chyle. The cocoanut oil fed had a molecular number of 7.3 and a melting point of 26°. The chyle fat had a molecular number of 19.1 and a melting point of 36°. The iodine number had increased.

The few results available on this point indicate that changes in the fats may be produced during absorption. Changes are probably greater than could be accounted for present in the normal fasting chyle.

In the work recorded below evidence is given of various changes in fats during absorption. It is probable that the intestine is able to regulate the composition of fats during absorption. The changes observed are toward the production of a body fat of the animal than the fat fed. This is probably purposive, since not only are they as different as could be produced by the fats present in the food but they vary in kind and degree with the nature of the food.

#### EXPERIMENTAL.

The changes above recorded consist of a raising of the melting point and raising of the iodine number of oleic acid. With the possible exception of Frank, no results are available which show a change in chyle fat in the reverse direction, *i.e.*, a lowering of the melting point and lowering of the iodine number of the chyle fat less liquid than the food fat.

<sup>13</sup> Raper: this *Journal*, xiv, p. 117, 1913.

<sup>14</sup> Bloor: *loc. cit.*

In the course of the study of another phase of fat absorption<sup>15</sup> it was observed that when olive oil (mixed with various hydrocarbons) was fed to dogs, the melting point of the chyle fatty acids was generally considerably higher than that of the olive oil fed, while the iodine number was lower. A further examination of the fat of these samples of chyle was made and the data which pertain to the present discussion are given below (Table I).

TABLE I.

EXP. NO.	MATERIAL FED	VOLUME OF CHYLE	WEIGHT OF CHYLE FAT	FATTY ACIDS FROM CHYLE FAT	
				M. P.	Iodine Number
		cc.	gms.		
I	Olive oil and hydro- carbon oil.....	165	1.2	30-32	86.8
II	Olive oil and hydro- carbon oil.....	65	0.8	30	79.4
III	Olive oil and hydro- carbon jelly.....	80	1.6	27	71.7
IV	Olive oil and hydro- carbon jelly.....	75	0.5	below 20°C.	80.9
V	Olive oil and hydro- carbon oil emulsi- fied.....	179	5.9	29.5	80.3
VI	Olive oil and hydro- carbon oil emulsi- fied.....	100	4.1	30	84.0

The fatty acids of the olive oil fed had a m.p. of about 16°, and an iodine number of 86.1°.

The chyle fatty acids obtained above had with one exception in each case a much higher melting point and a lower iodine number than the fatty acids of the olive oil fed. The unused fatty acids from these experiments were united, dissolved in ether, treated with bone black until the solution was nearly colorless, then the ether evaporated and the residue dried. The melting point of the combined fatty acids was 29.5°. The acids, on fractionation from chilled petroleum ether, yielded two fractions, the first of which was crystalline and amounted to 22.5 per cent of the whole. After repeated recrystallization it gave a melting point of about 56° (a fraction of constant m.p. could not be ob-

<sup>15</sup> Bloor: *loc. cit.*

tained), and an iodine number of 4.1. This fraction was probably a mixture of the higher saturated fatty acids.

The remaining portion, soluble in cold petroleum ether, after repeated chilling in small amounts of petroleum ether, until no more would separate, had an iodine value of 85.9 and a melting point of about 14°C. It was therefore nearly pure oleic acid.

There was obtained then after feeding olive oil whose fatty acids consisted of 96 per cent oleic acid, a chyle fat containing approximately 22.5 per cent of solid fatty acids and 77.5 per cent oleic acid and with an average melting point of 29.5°C.

As already noted, these results were obtained after feeding the olive oil in admixture with hydrocarbons and although none of the hydrocarbons were absorbed, they may have had an influence on the composition of the chyle fat. Further experiments on this point and to determine the nature of the glycerides obtained, are in progress.

In order to obtain more data regarding the changes in the fats during absorption and to determine whether there was any relationship between the amount of change and the nature (especially m.p.) of the fat fed, as well as to settle definitely whether the foreign fat found in the chyle was normally present in fasting chyle or was purposely added, experiments were conducted in which esters of pure fatty acids of various melting points were fed and the chyle fat examined.

*Feeding of pure fatty acid esters and collection of the chyle.*

Ethyl esters of stearic, palmitic and lauric acids were prepared by the action of their chlorides upon ethyl alcohol. The chlorides were prepared from pure fatty acids (mainly Kahlbaum's K preparations) by the method of Krafft and Bürger,<sup>16</sup> and in the preparation of the esters were added slowly with stirring to excess of absolute alcohol. The solution was allowed to stand over night, then poured into excess of water. After thorough washing the material was ready for use. The animals (dogs) were starved for forty-eight hours before the feeding. The esters, which were all liquid at body temperature, were given by stomach tube and the feeding was followed by about 50 grams of bread which had been rendered fat-free by boiling with alcohol.

<sup>16</sup> Krafft and Bürger: *Ber. d. deutsch. chem. Gesellsch.*, xvii, p. 1378, 1884.

The operations for the insertion of the cannula into the thoracic duct (for the earlier of which I am indebted to Doctor W. M. Marriott of this laboratory), were performed under ether anaesthesia. Special efforts were made to make the shock of operation as light as possible and to bring the animal after the operation into a condition as nearly normal as possible for the experiment. The operations were done with aseptic precautions making a small wound; and by the use of a paraffined cannula of narrow lumen (2 mm.), clotting was prevented without the use of special devices. As soon as the cannula was safely in the duct and the wound closed, the animal was removed to a padded table, covered warmly and allowed to recover from the anaesthetic. The return to consciousness was always followed by an improvement in the fat content of the chyle. Collection of chyle was continued as long as convenient. The animals rested quietly most of the time. If they became restless they were removed from the table and allowed to walk around for a while, after which they were returned to the table and generally went to sleep. Water was given as often as desired. As previously mentioned, the chyle was occasionally found to be flowing on the second day and another experiment could be made with the same animal. The cannula generally dropped out on the third day and the animal in almost all cases made a good recovery.

The chyle was collected in a vessel containing a little dry magnesium sulphate to prevent clotting and when collection was complete it was transferred to a separatory funnel, shaken well with ether and the mixture allowed to stand over night. The extracted chyle, now clear, was run off, evaporated to dryness on a water bath, powdered and again extracted by boiling out two or three times with ether. These ether extracts were added to the first and the whole evaporated to dryness. The essential points of the experiments are as follows:

**ETHYL STEARATE.** Dog, weight 10 kgm., a fat female, had been used in a similar experiment the day before. The chyle was still flowing and was clear. At 10 a.m. she was fed 7.3 grams of the pure ester of which a considerable portion was vomited shortly afterwards. The volume of chyle was not noted. Total chyle fat collected, 0.3 gram. The fat was saponified and the fatty acids separated from unsaponifiable matter and purified with bone black.

Melting point of the fatty acids, 45°C.

Iodine number, 56.21—containing therefore 62.5 per cent oleic acid.

Mean molecular weight, 285.

**ETHYL PALMITATE.** Experiment I. The dog, a female, weight 25 pounds, thin and active, was fed 20 grams of ethyl palmitate at 8.30 a.m. The operation was complete and collection was begun at 11.45. Collection was continued for ten hours with a total yield of chyle of 147 cc., containing 1.3 grams of fat—0.9 per cent.

Melting point of the fat, 55°.

Iodine number, 66.9.

Experiment II. Next morning the chyle was still flowing and clear, so another feeding of 20–25 grams of ester was given together with 50 grams of coagulated egg white, at 10.30 a.m. By 12 o'clock the chyle had begun to appear milky. Collection was then begun and continued until 7.45 p.m. Total chyle collected, 79 cc., containing 1.6 grams of fat—2 per cent.

Melting point of the fat, 57°C.

Iodine number, 52.3.

The chyle fat from the two palmitate experiments was united.

Average melting point, 56°. Average iodine number, 59.6.

It was fractionated from ether in the cold, yielding two main fractions of which the first and largest—1 gram—after several recrystallizations, yielded well formed crystals with a melting point of 61°C. and an iodine number of 7.6. It was probably nearly pure tripalmitin. The second fraction, from which no other substance could be separated was liquid at room temperature. Its iodine number was 62.5, corresponding to an oleic acid content of 69.4 per cent. (This value is suggestively close to that of a dioleopalmitin oleic acid, 65.7 per cent.)

**ETHYL LAURATE:** A female dog weighing 20 pounds, in fair condition, was fed 18 grams of ethyl laurate, together with 50 grams of fat-free bread and 10 grams of glycerin, at 8.30 a.m. Collection of chyle was begun at 1.15. From 1.15–3.15, 58 cc. containing but little fat were collected and treated separately (see below). At 3.15 the chyle was becoming richer in fat and continued of good color until 9.45 p.m., when collection was stopped. This portion (II), total 106 cc., was extracted separately (see below). Next morning the chyle was still flowing and of a good white color. Collection was begun again at 8.30. At about 11.00 a.m. it began to lose its white color and 9 grams more of ethyl laurate were fed through a stomach tube. Collection was continued until 9.45 p.m. The chyle from this period, total 131 cc., was treated separately (portion III). The extractions of the separate portions were made as in the other experiments.

Portion I. 58 cc.; total fat, 0.21 gram, 0.36 per cent; m.p. 22–24°C.

Portion II. 106 cc.; total fat, 1.82 grams, 1.71 per cent; m.p. 22–24°C.

Portion III. 131 cc.; total fat, 1.15 grams, 0.88 per cent; m.p. 30°C.

Iodine number, 56.35.

Portion IV. The chyle from the above three portions was evaporated to dryness, powdered and extracted with hot ether. Weight of extract 0.57 gram.

Total laurate chyle collected, 295 cc., containing 3.75 grams of fat—1.27

per cent. Iodine number of the whole chyle fat, 44. Attempts were made to fractionate this fat but without success.

The essential facts of the ester experiments are collected in table II. The results of Munk and Frank already cited (pp. 520-521) are added for comparison.

From the results given it may be seen:

1. That the amount of "oleic acid" in the chyle fat is generally much greater than could be accounted for by the fat of fasting chyle, accepting the average value 0.25 per cent already quoted (p. 521) and supposing it to be entirely oleic acid.

2. That there is a parallelism between the melting point of the fatty acids fed and the amount of "oleic acid" added—the higher the m.p. of the fatty acid, the more oleic acid.

3. That the fat of the fasting chyle is not always entirely, or even mainly, oleic acid, as may be seen from the laurate experiments. Since the chyle in portion I has the lowest fat content it should contain most of the fasting chyle fat, and if this fat were oleic acid, the iodine number should be highest of the three portions, while it is actually lowest.

4. That there is a marked similarity in the results of Frank's experiments with ethyl palmitate and those reported in this paper. The chyle fat of both yielded two main fractions, one of which was undoubtedly tripalmitin and the other with an iodine number and melting point close to those of mixed glycerides of palmitin and olein.

It seemed of interest to know also whether any change would be produced in a fat with an already high iodine number and low melting point. For this purpose cod liver oil, iodine number 148, was fed to a dog, the chyle collected and the chyle fat examined as in the earlier experiments.

Total chyle, 76 cc.

Fat of chyle, 1.05 gram—1.4 per cent.

Iodine number, 118.

The iodine number was reduced from 148 to 118 during the absorption.

Here for completeness may be mentioned again the results of experiments with cocoanut oil which have already been mentioned above (p. 522), as examples of a change of a somewhat different nature.

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Here for completeness may be mentioned again the results of experiments with cocoanut oil which have already been mentioned above (p. 522), as examples of a change of a somewhat different nature.

TABLE II.

MATERIAL FED	VOLUME OF CHYLE	CHYLE FAT		Iodine Num- ber	WEIGHT OF OLEIC ACID IN CHYLE FAT (CALC.)	WEIGHT OF ACID IN SAME VOL. OFFASTING CHYLE	REMARKS
		Weight	M. P.				
	cc.	grams.	deg. C.		grams.	grams.	
Ethyl stearate.....		0.3	45	56.2	0.2		Volume of chyle not noted.
Ethyl palmitate							
Exp. I.....	147	1.3	55	66.9	1.0	0.36	
Exp. II.....	79	1.6	57	52.3	0.97	0.19	
On Fractionation							
Fraction I.....			61	7.6			Probably tripalmitin, m.p., 65.5. Possibly di-oleopalmitin, iodine no. 59.2 (calc.).
Fraction II.....			below 20	62.5			
Ethyl laurate.							
Portion I.....	58	0.21	22-24	32.8	0.08	0.14	
Portion II.....	106	1.82	22-24	44.08	0.9	0.26	
Portion III.....	131	1.15	30	56.4	0.75	0.33	
Cetyl palmitate (Munk).....	1200	3.97	36		0.57*	1.2†	
Ethyl palmitate (Frank)							
Exp. 11.....	27	0.37	51	32.6	0.14	0.07	
Exp. 12.....	40	1.43			0.19	0.10	
On fractionation							
Fraction I.....			39	22.7			Values for an oleo-dipalmitin are m.p., 37-38; Iodine no. 31.4 (calc.). Probably tripalmitin.
Fractions II and IV (av.)			62	4.0			

\* The oleic acid was determined by separation as lead salt and the value is probably low.

† Calculated from his own determination of fat in human hunger chyle (0.1 per cent).

EXPERIMENT I (Raper): The coconut oil fed had an iodine number of 7.7, and a mean molecular weight of 212. The chyle fat collected had an iodine value of 19.1, and a mean molecular weight of 236.

EXPERIMENT II (Bloor): Coconut oil (fed together with hydrocarbon oil). Iodine number, 7.3; m.p., 26°; chyle fat iodine number, 24; m.p., 26°.

The changes in coconut oil consist in the addition of "oleic acid" without a change in the melting point.

#### SUMMARY AND CONCLUSIONS.

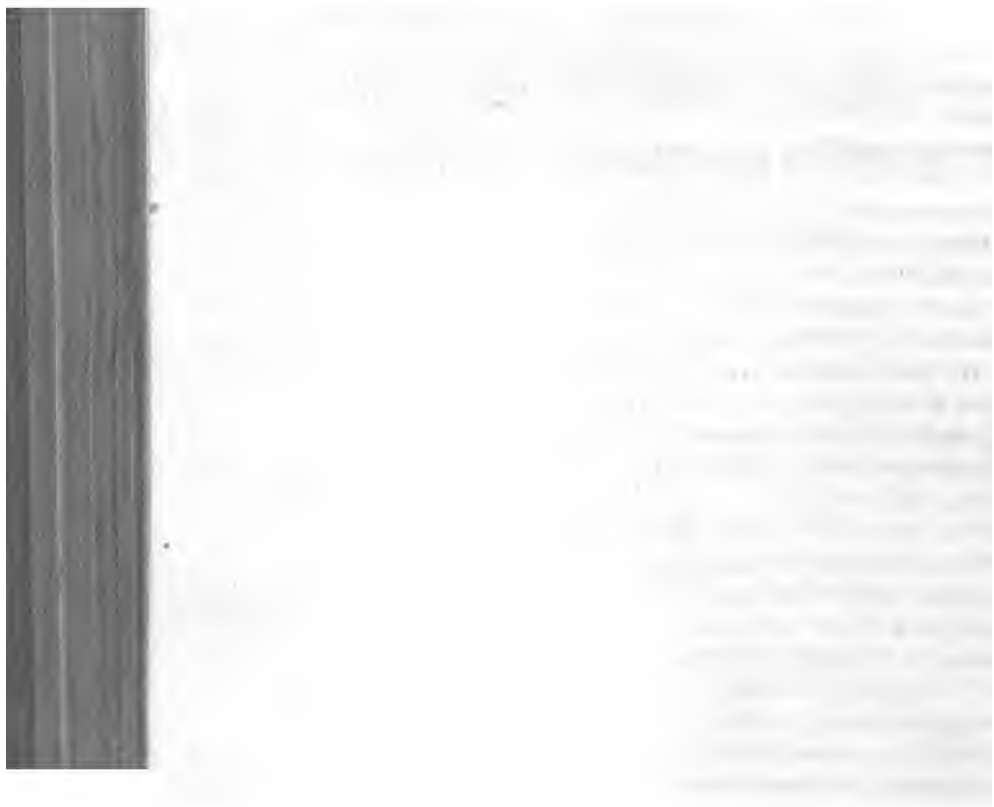
Evidence is presented of changes in fat during absorption as follows:

1. A lowering of the melting point of high melting point fats by the addition of an unsaturated fatty acid, probably oleic acid. The addition is proportional to the melting point of the fatty acid fed.
2. An elevation of the melting point and lowering of iodine number of a low melting point fat (olive oil) by the addition of saturated fatty acids.
3. Addition of "oleic acid" together with a change in the mean molecular weight of the fatty acids, without change of melting point in a fat which consists mainly of glycerides of saturated fatty acids (coconut oil).
4. Lowering of the iodine number of a fat (cod liver oil) which contains a large percentage of glycerides of highly unsaturated fatty acids.

The intestine appears to have the power to modify radically the composition of the fats during absorption. The changes are apparently purposive in that they vary in kind and degree with the nature of the fat fed and also show in general a *tendency* toward the production of a uniform chyle fat, presumably the characteristic body fat of the animal.

In the preceding paper of this series one reason was suggested for the peculiar mechanism of fat absorption—that it serves to exclude undesirable fat-like substances such as the petroleum hydrocarbons, etc. The observations presented above, suggest a second—that the mechanism serves to permit adaptive changes in the fats during absorption.

Work is being continued along similar lines.



## THE HEXONE BASES OF CASEIN.

By DONALD D. VAN SLYKE.

(From the Laboratories of the Rockefeller Institute for Medical Research, New York.)

(Received for publication, November 28, 1913.)

In our preliminary description of the method for analysis of proteins by determination of the chemical groups characteristic of the different amino-acids, we published an analysis of casein.<sup>1</sup> The results agreed quite well with those previously obtained by other authors with the Kossel method for determining the bases of proteins. Consequently, although our method was improved before its final publication, we did not repeat the casein analysis. The discrepancy, noted in the preceding article, between the free amino nitrogen of casein, and the lysine content previously determined, rendered a repetition of the nitrogen distribution in this protein desirable. We have, therefore, determined the bases by the method of Kossel and Patton, as modified by Osborne, Leavenworth, and Brautlecht,<sup>2</sup> and have also redetermined the bases and nitrogen distribution by our previously published method of group analysis.

The most significant differences between our present results and previous ones occur in the lysine. The percentages of the casein nitrogen previously found in the lysine were 6.66 to 7.24<sup>3</sup> by Kossel's method and 7.86 by our own. By exercising particular care in the Kossel method we have now obtained 9.36 per cent of the casein nitrogen in the form of lysine weighed as the analytically pure picrate. Our group determination method gave 10.3 per cent, and we believe that this figure is even more nearly correct, as the amount of lysine picrate which one can crystallize represents necessarily the minimum amount present. For arginine the results are practically the same, 7.4 to 7.8 per cent of the total nitrogen,

<sup>1</sup> *Ber. d. deutsch. chem. Gesellschaft.*, xliii, p. 3179, 1910.

<sup>2</sup> *Amer. Journ. of Physiol.*, xxiii, p. 183, 1908.

<sup>3</sup> *Ibid.*

as those previously obtained by both methods. The histidine results are a little higher than previously, but not to a marked extent.

The source of error in our own former results for lysine lay in the cystine determination. The lysine is estimated from the total amino nitrogen of the bases precipitated by phosphotungstic acid, after the cystine nitrogen has been subtracted. The cystine was estimated from the amount of organic sulphur precipitated with the bases. The original form of the method, however, made the cystine figures liable to error from the fact that sulphates could be dissolved from a glass flask used in one stage of the operation. Although this source was recognized<sup>4</sup> and a correction, determined from controls, attempted for it, the results for cystine were nevertheless much too high, those for lysine being consequently low. In the form to which the method was modified before being published in detail in this *Journal*,<sup>5</sup> the above source of error in the cystine and lysine determinations was eliminated.

In the determination by the picrate method, as usually performed, it appears that the most probable source of loss lies in the decomposition of lysine phosphotungstate with barium hydrate. In this operation one insoluble precipitate (lysine phosphotungstate) is transformed into another (barium phosphotungstate), a process the completeness of which is necessarily difficult to judge. Moreover, the bulky barium phosphotungstate has marked adsorptive properties, so that even skill and experience might not insure against loss from this source. In working out the details of the group determination method, we noticed that several per cent of the total nitrogen of the protein could be lost from the base fraction through adsorption or occlusion by the barium precipitate. We therefore made a practice of reducing this loss to a minimum by completely dissolving the phosphotungstates of the bases with alkali, and precipitating the barium phosphotungstate in a dilute solution.<sup>6</sup> In the more successful of our present determinations by Kossel's method we have dissolved the lysine phosphotungstate in ammonia and diluted the solution to a large volume before treating with barium hydrate.

<sup>4</sup> *Ber. d. deutsch. chem. Gesellsch.*, xliii, p. 3177, 1910, footnote.

<sup>5</sup> This *Journal*, x, p. 16, 1911.

<sup>6</sup> *Ibid*, x, p. 25, 1911.

*First analysis by Kossel's method.*

In this determination the casein was completely hydrolyzed with hydrochloric acid and all the bases were precipitated with phosphotungstic acid. The precipitate was decomposed with barium hydrate, using a large volume of solution and a mechanical stirrer to make the decomposition quantitative and keep loss by adsorption as low as possible. The bases were then separated by Osborne, Leavenworth, and Brautlecht's modification of Kossel and Patton's method. The details follow.

Forty grams of Merck's "Casein nach Hammarsten" were boiled thirty hours with 400 cc. of 20 per cent hydrochloric acid. The solution was then diluted to 1 liter, and three samples of 5 cc. each removed for Kjeldahl determinations. The amounts of  $\frac{N}{16}$  acid neutralized were 18.70, 18.67, and 18.65 cc., the average indicating 5.15 grams of nitrogen in the remaining 985 cc. of solution.

The latter was diluted with water to 2 liters, and the bases were precipitated with 150 grams of purified phosphotungstic acid. After two days the precipitate was filtered with suction and washed with a solution of 2.5 per cent phosphotungstic acid in 5 per cent sulphuric acid until the chloride reaction disappeared from the filtrate. The precipitate was then suspended in 5 liters of water and thoroughly stirred with a machine while an excess of barium hydrate solution was added. The stirring was then continued for about two hours. The filtrate from the barium phosphotungstate was concentrated in a vacuum, the ammonia being driven off in the process. The excess barium was then removed with carbon dioxide, and the solution concentrated to 1000 cc. Twenty-five cc. were removed for analyses, which gave a total nitrogen of 1.176 grams, or 22.8 per cent of the entire casein nitrogen, and an amino nitrogen of 0.754 gram, or 14.63 per cent.

The remaining 975 cc. of the solution, containing the basic portion of 5.02 grams casein nitrogen, were concentrated in a vacuum, and the histidine precipitated as described by Osborne, Leavenworth, and Brautlecht.

The histidine solution was brought to 100 cc. volume.

2.000 cc. for  $\text{NH}_2$  determination gave 2.51 cc. N gas at  $21^\circ$ , 774 mm.

10.00 cc. for Kjeldahl determination neutralized 14.92 cc. of  $\frac{N}{16}$  acid.

Amino nitrogen in histidine solution, 0.0723 gram.

Total nitrogen in histidine solution, 0.2090 gram = 4.16 per cent of total casein nitrogen.

Ratio (total nitrogen) : (amino nitrogen) = 2.89

Ratio calculated for histidine = 3.00

The arginine solution was also brought to 100 cc.

2.000 cc. for  $\text{NH}_2$  determination gave 3.41 cc. N gas at  $23^\circ$ , 764 mm.

5.00 cc. for Kjeldahl determination neutralized 13.44 and 13.50 cc. of  $\frac{N}{16}$  acid.

tained), and an iodine number of 4.1. This fraction was probably a mixture of the higher saturated fatty acids.

The remaining portion, soluble in cold petroleum ether, after repeated chilling in small amounts of petroleum ether, until no more would separate, had an iodine value of 85.9 and a melting point of about 14°C. It was therefore nearly pure oleic acid.

There was obtained then after feeding olive oil whose fatty acids consisted of 96 per cent oleic acid, a chyle fat containing approximately 22.5 per cent of solid fatty acids and 77.5 per cent oleic acid and with an average melting point of 29.5°C.

As already noted, these results were obtained after feeding the olive oil in admixture with hydrocarbons and although none of the hydrocarbons were absorbed, they may have had an influence on the composition of the chyle fat. Further experiments on this point and to determine the nature of the glycerides obtained, are in progress.

In order to obtain more data regarding the changes in the fats during absorption and to determine whether there was any relationship between the amount of change and the nature (especially m.p.) of the fat fed, as well as to settle definitely whether the foreign fat found in the chyle was normally present in fasting chyle or was purposely added, experiments were conducted in which esters of pure fatty acids of various melting points were fed and the chyle fat examined.

*Feeding of pure fatty acid esters and collection of the chyle.*

Ethyl esters of stearic, palmitic and lauric acids were prepared by the action of their chlorides upon ethyl alcohol. The chlorides were prepared from pure fatty acids (mainly Kahlbaum's K preparations) by the method of Krafft and Bürger,<sup>16</sup> and in the preparation of the esters were added slowly with stirring to excess of absolute alcohol. The solution was allowed to stand over night, then poured into excess of water. After thorough washing the material was ready for use. The animals (dogs) were starved for forty-eight hours before the feeding. The esters, which were all liquid at body temperature, were given by stomach tube and the feeding was followed by about 50 grams of bread which had been rendered fat-free by boiling with alcohol.

<sup>16</sup> Krafft and Bürger: *Ber. d. deutsch. chem. Gesellsch.*, xvii, p. 1378, 1884.



The operations for the insertion of the cannula into the thoracic duct (for the earlier of which I am indebted to Doctor W. M. Marriott of this laboratory), were performed under ether anaesthesia. Special efforts were made to make the shock of operation as light as possible and to bring the animal after the operation into a condition as nearly normal as possible for the experiment. The operations were done with aseptic precautions making a small wound; and by the use of a paraffined cannula of narrow lumen (2 mm.), clotting was prevented without the use of special devices. As soon as the cannula was safely in the duct and the wound closed, the animal was removed to a padded table, covered warmly and allowed to recover from the anaesthetic. The return to consciousness was always followed by an improvement in the fat content of the chyle. Collection of chyle was continued as long as convenient. The animals rested quietly most of the time. If they became restless they were removed from the table and allowed to walk around for a while, after which they were returned to the table and generally went to sleep. Water was given as often as desired. As previously mentioned, the chyle was occasionally found to be flowing on the second day and another experiment could be made with the same animal. The cannula generally dropped out on the third day and the animal in almost all cases made a good recovery.

The chyle was collected in a vessel containing a little dry magnesium sulphate to prevent clotting and when collection was complete it was transferred to a separatory funnel, shaken well with ether and the mixture allowed to stand over night. The extracted chyle, now clear, was run off, evaporated to dryness on a water bath, powdered and again extracted by boiling out two or three times with ether. These ether extracts were added to the first and the whole evaporated to dryness. The essential points of the experiments are as follows:

**ETHYL STEARATE.** Dog, weight 10 kgm., a fat female, had been used in a similar experiment the day before. The chyle was still flowing and was clear. At 10 a.m. she was fed 7.3 grams of the pure ester of which a considerable portion was vomited shortly afterwards. The volume of chyle was not noted. Total chyle fat collected, 0.3 gram. The fat was saponified and the fatty acids separated from unsaponifiable matter and purified with bone black.

Melting point of the fatty acids, 45°C.

grams of casein nitrogen, were submitted to the Osborne modification of Kossel and Patton's technique for separation of the two bases by precipitation of the histidine with mercuric sulphate.

The histidine solution was brought to 100 cc.

2.000 cc. for  $\text{NH}_3$  determination gave (1) 2.59 cc. N gas at  $17^\circ$ , 779 mm.;

(2) 2.69 cc. at  $25^\circ$ , 765 mm.

3.00-cc. portions for Kjeldahl determination required 5.00 cc. each of  $\frac{\text{N}}{10}$  acid.

Amino nitrogen in histidine solution, 0.0758 to 0.0752 gram; average, 0.0755 gram.

Total nitrogen in histidine solution, 0.2330 gram = 4.51 per cent of the casein nitrogen.

Ratio (total nitrogen) : (amino nitrogen) = 3.09

Ratio calculated for histidine = 3.00

The arginine solution was also brought to 100 cc.

2.000 cc. for  $\text{NH}_3$  determination gave 3.60 cc. N gas at  $17^\circ$ , 770 mm.

3.00 cc. for Kjeldahl required 8.60 to 8.65 cc., average 8.63 cc.,  $\frac{\text{N}}{10}$  acid.

Amino nitrogen in arginine solution, 0.1052 gram.

Total nitrogen in arginine solution, 0.4040 gram = 7.83 per cent of the casein nitrogen.

Ratio (total nitrogen) : (amino nitrogen) = 3.84

Ratio calculated for arginine = 4.00

From the filtrate of the first arginine-histidine precipitate the lysine was precipitated as phosphotungstate in the usual manner. The precipitate was redissolved in 2 liters of dilute ammonia and the phosphotungstic acid removed by addition of barium hydrate. The filtrate from the barium phosphotungstate was freed from ammonia by concentration under diminished pressure, from barium by means of carbon dioxide, and was then brought to 100 cc. Analysis showed that 0.605 gram of amino nitrogen was present. To 75 cc. of the solution one equivalent (3.75 grams) of picric acid was added, and the mixture was heated until solution was complete. After two days' standing 4.69 grams of lysine picrate, equivalent to 6.26 grams for the entire 100 cc. of solution, crystallized analytically pure. The amount of lysine nitrogen calculated from the picrate is 0.467 gram, or 8.62 per cent of the total nitrogen of the casein.

ANALYSIS: 0.1433 gram substance; 18.85 cc. N gas at  $19.5^\circ$ , 760 mm. freed by nitrous acid method.

	Calculated for $\text{C}_6\text{H}_{15}\text{O}_8(\text{NH}_2)_2\cdot\text{C}_6\text{H}_3\text{N}_3\text{O}_7$	Found:
Amino nitrogen.....	7.47	7.50

The filtrate from the above main crop of picrate was acidified with sulphuric acid, freed from picric acid with ether, and treated again with phosphotungstic acid in a volume of 100 cc. A second crop of lysine phosphotungstate was obtained, which eventually yielded 0.40 gram of pure lysine picrate.

ANALYSIS: 0.0327 gram substance; 4.35 cc. N gas at  $24^\circ$ , 760 mm., by the nitrous acid method with micro-apparatus.

	Calculated for $C_6H_{10}O_7(NH_2)_2 \cdot C_8H_8N_2O_7$	Found:
Amino nitrogen.....	7.47	7.42

This second crop of lysine brings the total lysine nitrogen up to 0.507 gram, equal to 9.36 per cent of the casein nitrogen.

*Analysis by the nitrogen distribution method.*

Ten grams of casein were boiled twenty-four hours with 200 cc. of 20 per cent hydrochloric acid.<sup>7</sup> The acid was driven off as completely as possible by concentrating under diminished pressure, and the solution was brought to 150 cc. Three samples of 5 cc. each required 35.05, 35.20, and 35.05 cc., an average of 35.10 cc., of  $\frac{N}{10}$  acid in Kjeldahl determinations. For the nitrogen distribution 50 cc. of the solution, containing 0.491 gram of nitrogen, were taken. The analysis was performed as described in the paper on the method.<sup>8</sup>

*Ammonia.* The amount of  $\frac{N}{10}$  acid neutralized was 36.00 cc., equivalent to 0.0504 gram of ammonia nitrogen.

*Melanine.* The amount of  $\frac{N}{10}$  acid neutralized was 4.50 cc., equivalent to 0.0063 gram of melanine nitrogen.

*Cystine.* The weight of barium sulphate was 0.0035 gram, equivalent to 0.0010 gram of cystine nitrogen in all.

*Arginine.* The volume of  $\frac{N}{10}$  acid neutralized was 6.50 cc., equivalent to 0.0364 gram of arginine nitrogen.

*Total nitrogen of the bases.* The amount of  $\frac{N}{10}$  acid neutralized in the Kjeldahl determination was 35.65 cc. Added to the amount neutralized in the arginine determination, this gives 42.15 cc., equivalent to 0.1181 gram of nitrogen, or 24.27 per cent of the total nitrogen of the casein.

*Amino-nitrogen of the bases.* Two-cc. portions of the solution of the bases gave, in the micro-apparatus, 5.02 cc. of nitrogen gas at 23°, 778 mm., and 4.94 cc. at 20°, 778 mm., indicating respectively 0.0722 and 0.0719 gram of amino nitrogen in the bases, equivalent to 14.6 per cent of the total nitrogen of the casein.

*Amino nitrogen of the filtrate.* Duplicates gave each 31.40 cc. nitrogen gas at 20°, 781 mm., equivalent to 0.274 gram of amino nitrogen.

*Total nitrogen of the filtrate.* The amounts of  $\frac{N}{10}$  acid neutralized were 36.65 and 36.90 cc., the average, 36.78 cc., indicating 0.309 gram of nitrogen in the filtrate.

The results are tabulated on the following page.

<sup>7</sup> Van Slyke: Conditions for Complete Hydrolysis of Proteins, this *Journal*, xii, p. 295, 1912.

<sup>8</sup> This *Journal*, x, p. 15, 1911.

	NITROGEN	TOTAL NITROGEN
	grams	per cent
Ammonia.....	0.0504	10.27
Melanine.....	0.0063	1.28
Cystine.....	0.0010	0.20
Arginine.....	0.0364	7.41
Histidine.....	0.0305	6.21
Lysine.....	0.0506	10.30
Amino nitrogen of filtrate.....	0.2740	55.81
Non-amino nitrogen of filtrate.....	0.0850	7.13
Total nitrogen recovered.....	0.4842	98.61

## SUMMARY.

The following figures for the bases of casein were obtained by the method of Kossel, and by the author's nitrogen distribution method. The figures represent percentages of the total nitrogen of the casein.

	KOSSEL'S METHOD		NITROGEN DISTRIBUTION METHOD (Uncorrected for solubility of bases)
	First Analysis	Second Analysis	
Histidine.....	4.16	4.51	6.21
Arginine.....	7.51	7.83	7.41
Lysine.....	8.70	9.36	10.30

It appears probable that low results for lysine (7 per cent) obtained previously by the Kossel method were due to adsorption or occlusion of lysine by barium phosphotungstate, a source of error which we attempted to avoid, especially in the second Kossel analysis. That the lysine crystallized as picrate represents the entire amount present is improbable, and the lysine content obtained by the nitrogen distribution method is doubtless more nearly correct.

From the data in this and previous papers,<sup>9</sup> which permit a comparison of results by the two methods, it appears that the nitrogen distribution method is somewhat more reliable than the Kossel method for lysine determination in proteins, that both methods are quite accurate for arginine, and that the Kossel-Patton method, as modified by Osborne, Leavenworth, and Brautlecht, gives more consistent results for histidine.

<sup>9</sup> This *Journal*, x, p. 16, 1911.

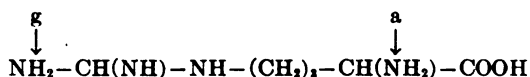
## THE NATURE OF THE FREE AMINO GROUPS IN PROTEINS.

BY DONALD D. VAN SLYKE AND FREDERICK J. BIRCHARD.

(From the Laboratories of the Rockefeller Institute for Medical Research, New York.)

(Received for publication, November 28, 1913.)

The presence of basic groups in the proteins has long been assumed because of the ability of the proteins to neutralize acids. The specific nature of these basic groups appears to have been first indicated by work on the protamines in Kossel's laboratory. These simplest proteins are all unusually rich in one or more of the hexone bases, arginine, histidine, and lysine, and are also markedly basic, forming salts of constant composition with sulphuric acid. Goto<sup>1</sup> found that clupeine, which contains a large amount of arginine, binds approximately 1 equivalent of sulphuric acid for each molecule of arginine. Arginine contains two amino groups, one ( $\alpha$ ) in the  $\alpha$ -position to the carboxyl group, the



other ( $g$ ) in the guanidine nucleus, and it was uncertain which of these was free in the clupeine molecule. The point was settled by Kossel and Cameron<sup>2</sup> in favor of the guanidine group. They nitrated the free amino groups of clupeine under conditions which avoided hydrolysis of the protein. The nitroclupeine was then hydrolyzed, and nitroarginine obtained from it. The amino group in this nitroarginine could be determined by the nitrous acid method. As the guanidine group does not react with nitrous acid, the amino group freed by hydrolysis was evidently the  $\alpha$ -group. The other

<sup>1</sup> *Zeitschr. f. physiol. Chem.*, xxxvii, p. 114, 1903.

<sup>2</sup> *Ibid.*, lxxvi, p. 457, 1912.

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amino group, free in the intact protamine, is therefore in the guanidine nucleus.

Other evidence indicated that, in some proteins at least, one of the two amino groups of lysine,  $\text{NH}_2(\text{CH}_2)_4\text{CH}(\text{NH}_2)\text{COOH}$ , is also free. Skraup<sup>3</sup> found that after casein, gelatin, and serum globulin had been treated with nitrous acid, no lysine could be obtained from them. Similar results were obtained by Levites.<sup>4</sup> Van Slyke demonstrated in edestin and egg albumin the presence of definite amounts of free amino nitrogen, which, though small, could be determined by the nitrous acid method, and pointed out the fact that, considering the results of Levites and Skraup, it was probable that one of the amino groups of lysine furnished a large part of the free amino nitrogen of the proteins.<sup>5</sup> Since then, having determined the lysine contents of a number of protein preparations in this laboratory, we have also determined their free amino nitrogen. The results, reported in detail in the present paper, were published in a preliminary abstract in May, 1912.<sup>6</sup> They showed that the free amino nitrogen of the native proteins is approximately one-half of the lysine nitrogen, indicating that one of the two amino groups of lysine is free in the protein molecule. At the time of our preliminary report the paper by Kossel and Cameron also appeared.<sup>7</sup> They settled the location of the free amino group of arginine in clupeine, as mentioned above, and also showed that clupeine, which contains no lysine, gave off no nitrogen when treated with nitrous acid to determine the free amino groups. Cyprinine and sturine, which contain lysine, showed considerable free amino nitrogen. Later Kossel and Gawrilow<sup>8</sup> performed the formol titration of Sørensen on hordein, zein, and several protamines, and found that the protamines containing lysine revealed amino groups by the formol method, while those which contained none, as well as zein, which also contains none, revealed no amino groups. No quantitative relations were ascer-

<sup>3</sup> *Ann. d. Chem.*, cceli, p. 379, 1906.

<sup>4</sup> *Biochem. Zeitschr.*, xx, p. 224, 1909.

<sup>5</sup> *This Journal*, ix, p. 196, 1911.

<sup>6</sup> *Proc. Soc. Exp. Biol. and Med.*, May 15, 1912.

<sup>7</sup> *Loc. cit.*

<sup>8</sup> *Zeitschr. f. physiol. Chem.*, lxxxi, p. 274, 1912.

tained, however, between the lysine and free amino nitrogen contents of the proteins.<sup>9</sup>

In our preliminary report we gave determinations of free amino nitrogen on several native proteins, and quoted those previously published from this laboratory on proto- and heteroalbumose. The native proteins showed, as stated above, free amino nitrogen equal to approximately half their lysine nitrogen. In the hetero- and protoalbumose we found more of the total nitrogen in the form of free amino nitrogen than is calculated by halving the lysine nitrogen. This result was to be expected, as the hydrolytic cleavage from which the albumoses result sets free  $\alpha$ -amino groups from the peptide linkings into which they are condensed in the native proteins.

We have in the meantime analyzed, in addition, gliadin, repeated with present technique the amino determination performed several years ago on the albumoses, and confirmed the results reported for the native proteins, except casein. The amount of free amino nitrogen in casein (3.4 per cent of the total nitrogen) was, through an error, calculated too low. The correct figure is 5.5 per cent. The former, incorrect figure was almost exactly one-half the lysine nitrogen previously determined in casein. The lysine content of casein had been determined some years before by the picrate method, but in the case of casein, unlike most of the other proteins, the estimation had not been checked by our group determination method in its present form. The hexone base content of casein was, therefore, carefully redetermined by both the Kossel and the group determination methods. The results are recorded in the preceding paper. The correct lysine nitrogen was found to be, as in the other proteins, nearly twice the free amino nitrogen.

For the hemocyanin preparation we thank Dr. C. L. Alsberg of the Bureau of Chemistry; for the hemoglobin, Dr. Butterfield of this Institute, and for the zein, Dr. Thomas B. Osborne of New Haven.

<sup>9</sup> "Im allgemeinen scheinen die lysinreicheren Protamine auch reicher an formoltitrierbarem Stickstoff zu sein, doch sind die bisher vorliegenden Analysen noch nicht zahlreich genug, um hierüber zu entscheiden."

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### EXPERIMENTAL.

*Methods.* The proteins were brought into solution in 2 to 4 per cent concentration, using when necessary acetic acid or sodium carbonate in the cold to assist the process. There was no evidence of the occurrence of any hydrolysis during the preparation of the solutions. These were analyzed immediately after they were made up. When they were allowed to stand several hours, only slight increases in the amino nitrogen were noted. All determinations were made in the standard size amino apparatus described in this *Journal*, xii, p. 275, or in the micro-apparatus described in xvi, p. 121. The mixtures were shaken constantly with a motor during each determination, octyl alcohol being used to prevent foaming. The proteins, or deaminized proteins, are precipitated as soon as they are mixed with the nitrous acid solution. When the mixture is kept well stirred by shaking, however, the precipitation does not appear to influence the results, which were uniformly definite and constant. That the amino nitrogen thus determined represents amino groups free in the protein, none of the latter being hydrolyzed by the nitrous acid, is indicated by two facts:

1. Peptides of varied composition and containing up to fourteen amino-acids in the molecule have been analyzed by our method and found to give theoretical results.<sup>10</sup>

2. The evolution of nitrogen is complete inside of twenty or thirty minutes, following practically the course found in analysis of lysine,<sup>11</sup> of which the  $\omega$ -NH<sub>2</sub> group reacts somewhat more slowly than the  $\alpha$ -groups of the amino-acids in general. We do not believe that any part of the amino nitrogen determined comes from acid amide groups in the protein molecule. As determined by analysis of asparagine and acetamide, acid amide groups give off no nitrogen at all when treated with nitrous acid under the conditions of the determination.

*Casein.* Three grams of Hammarsten casein (air-dried) were dissolved in 100 cc. of water with 0.375 gram of sodium carbonate. This amount of carbonate is sufficient to dissolve the casein without rendering the solution alkaline, and autohydrolysis occurs only at a very slow rate.

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<sup>10</sup> Abderhalden and Van Slyke: *Zeitschr. f. physiol. Chem.*, lxxiv, p. 505, 1911.

<sup>11</sup> Van Slyke: this *Journal*, xii, p. 275, 1912.



Kjeldahl nitrogen: 5-cc. portions; 14.80 cc. of  $\frac{N}{10}$  HCl (average of 3 determinations), indicating 8.29 mgm. of nitrogen in the 2 cc. of solution used for amino determination in the micro-apparatus. In this table the uncorrected as well as corrected results are given to show the magnitude of the correction for reagents, and its variation with the reaction time. In the subsequent tables only corrected results are given.

*Amino nitrogen: 2-cc. portions.*

DURATION OF REACTION WITH NITROUS ACID	NITROGEN GAS EVOLVED		TEMPERATURE	PRESSURE	AMINO N	PER CENT OF TOTAL N AS FREE NH <sub>2</sub>
	Volume read	Corrected for reagents				
min.	cc.	cc.	deg. C.	mm.	mgm.	
10	0.86	0.76	22	756	2.130	5.14
15	0.92	0.80	23	756	2.230	5.38
20	0.96	0.82	23	756	2.285	5.51
30	0.98	0.82	23	756	2.285	5.51

After the solution had stood forty-eight hours at room temperature the proportion of nitrogen as free NH<sub>2</sub> had increased to 5.84 per cent (thirty-minute reaction), indicating an appreciable, but very slow, autohydrolysis.

*Gelatin.* Kjeldahl nitrogen: 10-cc. portions; 24 cc.  $\frac{N}{10}$  HCl. Total nitrogen in 10 cc., 33.6 mgm.

*Amino nitrogen: 10-cc. portions.*

DURATION OF REACTION	N GAS	TEMPERATURE	PRESSURE	AMINO N	PER CENT OF TOTAL N AS FREE NH <sub>2</sub>
min.	cc.	deg. C.	mm.	mgm.	
10	1.80	23	762	1.026	3.05
30	1.90	28	762	1.048	3.12
30	1.90	28	762	1.048	3.12

*Ox hemoglobin.* Solution I. Kjeldahl nitrogen: 10-cc. portions; 12.93 cc.  $\frac{N}{10}$  HCl; 18.10 mgm. N. Solution II. Kjeldahl nitrogen: 10-cc. portions; 10 cc.  $\frac{N}{10}$  HCl; 14.01 mgm. N.

*Amino nitrogen: 10-cc. portions.*

NO.	DURATION OF REACTION	N GAS	TEMPERATURE	PRESSURE	AMINO N	PER CENT OF TOTAL N AS FREE NH <sub>2</sub>
	min.	cc.	deg. C.	mm.	mgm.	
Ia	10	1.80	18	760	1.033	5.70
Ib	10	1.80	18	760	1.033	5.70
IIa	30	1.50	26	758	0.825	5.89
IIb	30	1.60	26	758	0.881	6.29

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*Edestin.* Solution I. Kjeldahl nitrogen: 10-cc. portions; 24 cc.  $\frac{N}{10}$  HCl; 37.60 mgm. N. Solution II. Kjeldahl nitrogen: 10-cc. portions; 19.33 cc.  $\frac{N}{10}$  HCl; 27.06 mgm. N.

*Amino nitrogen: 10-cc. portions.*

NO.	DURATION OF REACTION	N GAS	TEMPERATURE	PRESSURE	AMINO N	PER CENT OF TOTAL N AS FREE NH <sub>2</sub>
	min.	cc.	deg. C.	mm.	mgm.	
Ia	10	1.00	20	762	0.570	1.69
Ib	10	1.00	20	762	0.570	1.69
IIa	30	0.80	25	766	0.448	1.65
IIb	30	0.90	26	758	0.495	1.83

*Hemocyanin.* The substance was prepared for analysis by grinding to a fine powder, then rubbing it up with 5 per cent sodium carbonate until a colloidal solution was obtained. This was poured into an excess of glacial acetic acid, and the mixture formed a clear solution, which was diluted with water.

Kjeldahl nitrogen: 5-cc. portions; 20.4 cc.  $\frac{N}{10}$  HCl. Total nitrogen in 10 cc., 57.12 mgm.

Amino nitrogen: 10 cc. of solution; N gas in thirty minutes, 4.20 cc. at 20°, 765 mm.; amino N, 2.41 mgm.; per cent total nitrogen as amino nitrogen, 4.28.

*Zein.* The substance was dissolved in glacial acetic acid.

Kjeldahl nitrogen: 10-cc. portions; 24.36 cc.  $\frac{N}{10}$  HCl; total N, 34.00 mgm.

Amino nitrogen: 10 cc. of solution gave in thirty minutes the same volume of gas as 10 cc. of glacial acetic acid alone in the control determination. Amino nitrogen not present.

*Gliadin.* Two grams of gliadin were rubbed up with 5 cc. of glacial acetic acid, the turbid solution diluted to 40 cc. with water, and cleared by centrifugalizing.

Kjeldahl nitrogen: 5-cc. portions; 22.65 cc.  $\frac{N}{10}$  HCl, indicating 12.70 mgm. of nitrogen in 2 cc.

*Amino nitrogen: 2-cc. portions.*

DURATION OF REACTION	N GAS	TEMPERATURE	PRESSURE	AMINO N	PER CENT OF TOTAL N
min.	cc.	deg. C.	mm.	mgm.	
15	0.21	21	764	0.12	0.94
30	0.25	21	764	0.14	1.10

*Heteroalbumose* (from Witte peptone), dissolved in 0.5 per cent Na<sub>2</sub>CO<sub>3</sub> solution.

Kjeldahl determination: 5-cc. portions; 11.65 cc.  $\frac{N}{10}$  HCl, indicating 6.52 mgm. N in 2 cc. solution.

*Amino nitrogen: 2-cc. portions.*

DURATION OF REACTION	N GAS	TEMPERATURE	PRESSURE	AMINO N	PER CENT OF TOTAL N
min.	cc.	deg. C.	mm.	mgm.	
15	0.87	24	762	0.488	7.48
30	0.94	24	762	0.526	8.06

*Protoalbumose* (from Witte peptone). Solution I. 0.750 gram albumose dissolved in 25 cc. water. Kjeldahl nitrogen: 5 cc. portions; 15.44 cc.  $\frac{N}{10}$  HCl, indicating 8.65 mgm. of nitrogen in 2 cc.

Solution II. 0.750 gram albumose dissolved in 25 cc. of 0.5 per cent  $\text{Na}_2\text{CO}_3$  solution. Kjeldahl nitrogen: 5-cc. portions; 15.49 cc.  $\frac{N}{10}$  HCl, indicating 8.67 mgm. of nitrogen in 2 cc.

*Amino determinations: 2-cc. portions.*

SOLUTION	DURATION OF REACTION	N GAS	TEMPERATURE	PRESSURE	NH <sub>3</sub> -N	PER CENT OF TOTAL N	CORRECTED FOR NH <sub>3</sub> IN PREPARATION
	min.	cc.	deg. C.	mm.	mgm.		
I	5	1.34	19	766	0.773	8.94	
II	5	1.32	18	764	0.760	8.77	
I	15	1.70	24	762	0.953	11.03	
II	15	1.53	18	764	0.881	10.16	
I	30	1.74	24	762	0.975	11.28	9.93
II	30	1.67	18	764	0.965	11.13	9.78

The final, thirty-minute results, are no higher when sodium carbonate is used to assist solution (solution II) than when pure water is used (solution I). This fact shows that the dilute sodium carbonate solution had no immediate hydrolyzing effect on the protein.

Unlike the heteroalbumose, the preparation of protoalbumose<sup>12</sup> contained an appreciable amount of free ammonia. Portions of 0.500 gram, used for determination of free ammonia by vacuum distillation from solution made alkaline with calcium hydrate, gave 0.75 and 0.80 cc. of  $\frac{N}{10}$  ammonia, equal to 1.46-1.55 per cent of the total nitrogen. In thirty minutes approximately 90 per cent of the nitrogen of ammonia is given off in the amino determination. The free amino nitrogen determined should therefore be reduced by 1.35 per cent of the total to correct for the ammonia.

The figures for free amino nitrogen in hetero- and protoalbumose are higher than those given in the papers from this laboratory on the composi-

<sup>12</sup> Levene, Van Slyke, and Birchard: *this Journal*, viii, p. 269, 1910.

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tion of the albumoses.<sup>13</sup> The earlier determinations were run for intervals of only five minutes. These suffice for all the other amino-acids, but are not long enough for complete decomposition of the  $\omega$ -NH<sub>2</sub> group of lysine.

### Summary of results.

In the following table the average results of the complete (thirty-minute) determinations are collected. The figures obtained by halving the lysine nitrogen are given for comparison.

PROTEIN ANALYZED	PER CENT OF TOTAL NITROGEN AS	
	Free amino N	One-half the lysine N
<i>A. Native proteins:</i>		
Hemoglobin.....	6.00	5.80*
Casein.....	5.51	5.15†
Hemocyanin.....	4.30	4.25‡
Gelatin.....	3.12	3.15‡
Edestin.....	1.80	1.90‡
Gliadin.....	1.10	0.38‡
Zein.....	0.00	0.00§
<i>B. Albumoses from fibrin:</i>		
Heteroalbumose.....	8.06	5.15**
Protoalbumose.....	9.86	4.80**

\* Unpublished result.

† Van Slyke: Preceding paper.

‡ Van Slyke: *This Journal*, x, p. 16, 1911.

§ Osborne and Jones: *Ergeb. d. Physiol.*, x, p. 99, 1910.

\*\* Levene, Van Slyke and Birchard: *This Journal*, x, p. 57, 1911.

### CONCLUSIONS.

In all the native proteins investigated the amount of free amino nitrogen is equal to one-half the lysine nitrogen, no deviation exceeding the limit of experimental error of the amino and lysine determinations being found in any case with the possible exception of gliadin, in which the difference is 0.7 per cent. The period required for complete reaction of the proteins with nitrous acid (thirty minutes) is longer than that required by the  $\alpha$ -amino groups (three to four minutes), but corresponds to that found for

<sup>13</sup> Levene, Van Slyke, and Birchard: *this Journal*, viii, p. 272, 1910; x, p. 59, 1911.

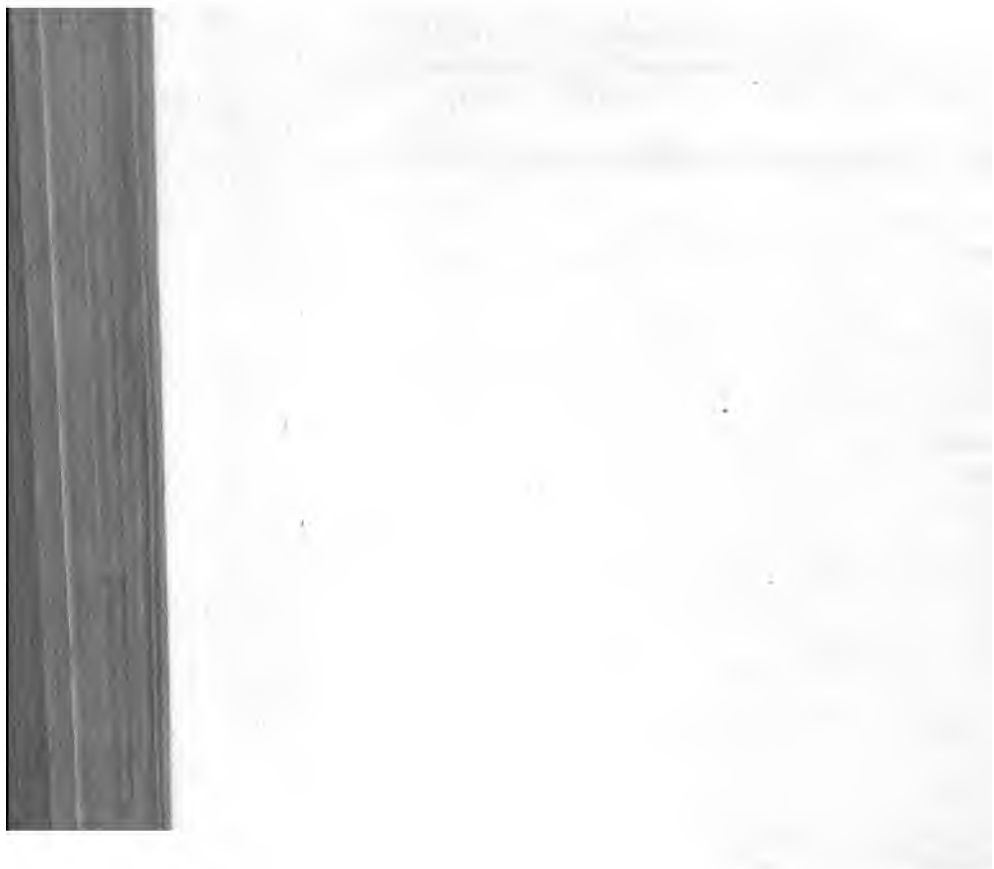
lysine, with an  $\omega$ -amino group free. The facts support the following conclusions.

1. One of the two amino groups of lysine, the  $\omega$ -group, exists free in the protein molecule.

2. This group represents, within at most a fraction of a per cent of the protein nitrogen, the entire amount of free  $\text{NH}_2$  determinable in the native proteins by the nitrous acid method.<sup>14</sup> The  $\alpha$ -amino groups, which constitute the remaining and greater part of the free amino nitrogen found after complete hydrolysis, are, in the intact protein molecule, practically all condensed into peptide linkings.

3. With the primary albumoses the relations are different. The free  $\text{NH}_2$  in hetero- and protoalbumose exceeds half the lysine nitrogen by 3 and 4.8 per cent, respectively, of the total protein nitrogen, indicating that an appreciable portion of the  $\alpha$ -amino groups is uncovered in even the primary digestion products.

<sup>14</sup> Osborne, Leavenworth and Brautlecht have demonstrated the probable presence of the acid amide groups of glutamine and asparagine in the protein molecule (*Amer. Journ. of Physiol.*, xxiii, p. 180). Acid amide groups, however, like the guanidine nucleus of arginine, give off none of their nitrogen when treated with nitrous acid, and consequently are not determined by our method.



## ON SPHINGOSINE.

SECOND PAPER.

### THE OXIDATION OF SPHINGOSINE AND DIHYDROSPHINGOSINE.

By P. A. LEVENE AND C. J. WEST.

(From the Laboratories of the Rockefeller Institute for Medical Research,  
New York.)

(Received for publication, December 1, 1913.)

In 1911 Levene and Jacobs<sup>1</sup> announced the first information regarding the chemical structure of sphingosine. They regarded the substance as a dihydroxy derivative of an unsaturated primary amine. Later Thierfelder and Thomas<sup>2</sup> corroborated some of these conclusions. Levene and Jacobs<sup>3</sup> in their full publication on sphingosine stated that further work on the structure of the base was in progress in this laboratory. It was made clear that the respective positions of the hydroxy groups, of the amino group and of the double bond were under investigation.

The progress of the work was not quite so rapid as expected for the reason that the reduction of dihydrosphingosine into the primary amine offered unexpected difficulties. In the course of these reduction experiments unexpected substances were obtained which may prove of considerable interest and the study of which somewhat delayed the completion of the work.

While this work was in progress there appeared during the course of the present summer a publication by Lapworth<sup>4</sup> on the structure of sphingosine. The investigations of Lapworth were begun in 1910, but the experiments reported in his publication are apparently based on the knowledge of the structure of the substance furnished by the work of Levene and Jacobs. Lapworth demonstrated that on oxidation of the base with chromium tri-

<sup>1</sup> Levene and Jacobs: *this Journal*, xi, p. xxix, 1912.

<sup>2</sup> Thierfelder and Thomas: *Zeitschr. f. physiol. Chem.*, lxxvii, p. 511, 1912.

<sup>3</sup> Levene and Jacobs: *this Journal*, xi, p. 547, 1912.

<sup>4</sup> Lapworth: *Journ. Chem. Soc.*, ciii, p. 1029, 1913.

oxide a tridecylic acid was obtained, which he regarded as the normal acid and for this reason argued that in sphingosine the carbon atoms are linked in a straight chain.

Our work on the structure of sphingosine is as yet not completed but we wish to present some of the results of our experiments, particularly in view of the publication of Lapworth. Only those experiments will be discussed in the present communication which deal with the oxidation of sphingosine and dihydrosphingosine. On oxidation of the unsaturated base a tridecylic acid was obtained, while the reduced base under the same conditions of experimentation gave rise to a pentadecylic acid. Regarding the structure of the carbon chains of the two acids, we as yet have no definite information. On the basis of the melting points of the acids one seems justified in concluding that the carbon atoms are not linked in a straight chain, since the normal tridecylic acid has a melting point of  $43^{\circ}$ ,<sup>5</sup> the normal pentadecylic acid melts at  $53^{\circ}$ , while the melting points of our two acids were  $47-48^{\circ}$  and  $60-61^{\circ}$  respectively. On this point our results do not agree with those of Lapworth. It will be the aim of the future work to establish the exact structure of the carbon chains of these two acids.

Our results, however, are important principally for the reason that through them the position of the double bond is made clear, namely, between the fourth and fifth carbon atoms (from the right end); and further, that through them the possibilities of the position of the two hydroxy and of the amino groups were limited to the carbon atoms 1, 2 and 3. On the ground of this one may express the structure of sphingosine approximately as follows:



The nature of the carbon chain of the part from 5 to 17 is not yet clear and the distribution of the hydroxy and amino groups on the carbon atoms 1-3 is not yet determined. However if the position of a hydroxyl or of an amino group had been removed further than the third carbon atom, then on oxidation of the dihydrobase, instead of the pentadecylic acid, a hydroxy- or an amino-acid should have been formed.

<sup>5</sup> Le Sueur: *Journ. Chem. Soc.*, lxxxvii, p. 1905, 1905.



## EXPERIMENTAL PART.

Our first experiments in the study of the oxidation products of sphingosine were attempts to repeat the work of Lapworth. We added an excess of chromic acid to a glacial acetic acid solution of sphingosine sulphate, keeping the reaction at about 70° on the water bath, the lowest temperature which caused effervescence. The excess of chromic acid was then reduced with sulphur dioxide and the reaction product distilled with steam. Only a very small amount of solid distillate passed over with the steam, 5 grams of sphingosine sulphate giving not more than 0.5 gram of crude acid. There remained in the flask an oily, green residue, soluble in ether, which we attempted to reoxidize, with little success, however. This green product was then boiled with concentrated hydrochloric acid, but it did not give a colorless acid as stated by Lapworth. Since then we have found that this oil contains most of the reaction product which may be obtained by distillation in vacuum, but have not repeated the work under exactly these conditions. In view of this we do not wish to condemn the method until we have tried it again.

After this we tried many conditions of oxidation and finally found the following to be the most suitable. Three grams of sphingosine sulphate were dissolved in about 50 cc. glacial acetic acid, warmed on a boiling water bath to about 85–90°, and a warm solution of 12 grams of chromic acid in 120 cc. glacial acetic acid slowly dropped into it, the flask being shaken quite frequently. After the addition of the acid, the reaction product was diluted with water and distilled with steam until nearly all of the acetic acid had been removed. A small amount of solid acid distilled over with the steam, which, after being dried in ethereal solution, weighed 0.350 gram. The main part of the acid was found in the oily, green residue, which floated on the surface of the dilute acetic acid in the flask. This solidified upon cooling in the ice box, and after drying the ethereal solution weighed 1.03 grams. It contained about 10 per cent ash. The theory from 3 grams sphingosine sulphate is about 1.9 grams.

The green product, when dried in vacuum, gave the following numbers upon analysis, which, calculated on an ash-free substance, indicated the presence of a  $C_{13}$  acid.

0.1170 gram of the substance gave 0.2790 gram  $\text{CO}_2$  and 0.1088 gram  $\text{H}_2\text{O}$ .  
The ash weighed 0.0125 gram.

	Calculated for $\text{C}_{13}\text{H}_{25}\text{O}_2$	Found:
C.....	72.80	73.17
H.....	12.20	11.82

This dried product was then distilled in vacuum, when a colorless distillate was obtained, which, when distilled twice, was found to be a pure tridecylic acid.

0.1222 gram substance gave 0.3248 gram  $\text{CO}_2$  and 0.1338 gram  $\text{H}_2\text{O}$ .

	Calculated for $\text{C}_{13}\text{H}_{25}\text{O}_2$	Found:
C.....	72.80	72.58
H.....	12.20	12.25

*Molecular weight estimation.* 0.5300 gram of the acid, dissolved in absolute methyl alcohol and benzene, required 24.8 cc.  $\frac{N}{10}$  NaOH for neutralization, using phenolphthalein as an indicator.

	Calculated for $\text{C}_{13}\text{H}_{25}\text{O}_2$	Found:
Molecular weight.....	214	214

The acid on the last distillation boiled between  $190\text{--}200^\circ$ , a glass water pump being used. The colorless product melted at  $46\text{--}47^\circ$ , when cooled and reheated at  $46\text{--}47^\circ$  and after standing over night and carefully heated,  $47\text{--}47.5^\circ$ , the last to be considered as the correct melting point. When recrystallized from dilute acetone the melting point was not changed.

Since the melting point of the acid was higher than that found by Lapworth ( $39\text{--}40^\circ$ ) and differed by  $4\text{--}5^\circ$  from the melting point of the normal tridecylic acid ( $43^\circ$ ), we repeated the oxidation on a larger quantity of sphingosine sulphate. The acid, after the second distillation, melted fairly sharply at  $42\text{--}43^\circ$ , after recrystallization from pure formic acid at  $42\text{--}43^\circ$ , but when recrystallized from about 90 per cent acetone, the melting point was raised to  $46.5\text{--}47.5^\circ$  as found above.

#### *Oxidation of dihydrosphingosine.*

A preliminary experiment was also carried out using dihydrosphingosine. Three grams of dihydrosphingosine sulphate, dissolved in about 125 cc. glacial acetic acid, were treated with 12 grams of chromic acid dissolved in 120 cc. glacial acetic acid and the reac-

tion product worked up as given above. The green mixture was distilled in vacuum and the colorless acid twice redistilled. The product thus obtained melted at 48–49°. When recrystallized from a little dilute acetone it melted at 60–61°. The normal pentadecylic acid, which we prepared from  $\alpha$ -hydroxypalmitic acid, melted at 53°. <sup>6</sup>

0.1288 gram substance gave 0.3506 gram CO<sub>2</sub> and 0.1410 gram H<sub>2</sub>O.

0.1159 gram substance gave 0.3154 gram CO<sub>2</sub> and 0.1306 gram H<sub>2</sub>O.

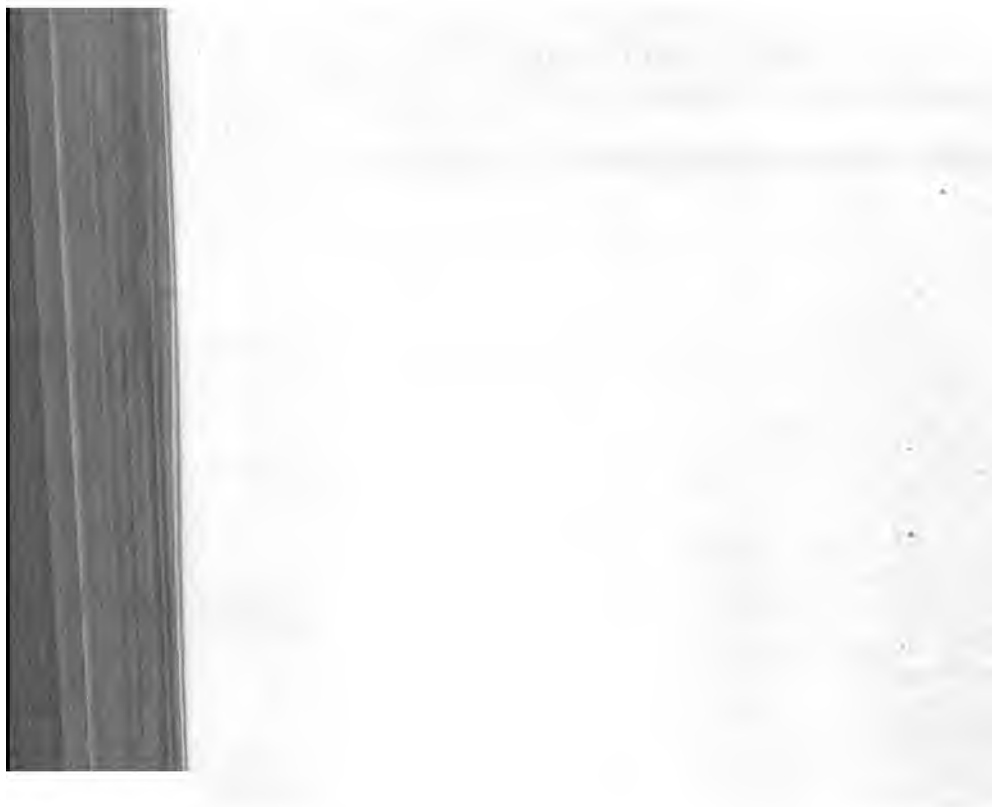
0.5282 gram substance required 21.72 cc.  $\frac{N}{10}$  NaOH for neutralization.

	Calculated for C <sub>15</sub> H <sub>32</sub> O <sub>2</sub>	Found:	
C.....	74.40	74.24	74.28
H.....	12.40	12.25	12.65
Molecular weight.....	242	243.3	

It may be mentioned that Liebermann<sup>7</sup> describes a pentadecylic acid, melting at 59–60°, which he obtained by the oxidation of cocceryl alcohol. Whether our acid is identical with this acid has not yet been determined.

<sup>6</sup> Levene and West: this *Journal*, xvi, p. 475, 1914.

<sup>7</sup> Liebermann and Bergami: *Ber. d. deutsch. chem. Gesellsch.*, xx, p. 959, 1887.



## ON THE ACTION OF LEUCOCYTES AND OF KIDNEY TISSUE ON AMINO-ACIDS.

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New York.)

(Received for publication, December 1, 1913.)

Since the publication of Lang<sup>1</sup> in 1904 it was generally accepted that through the action of tissues amino-acids underwent a deamination which led to the formation of the corresponding hydroxy-acids. It was also considered probable, on the basis of the work of Neubauer,<sup>2</sup> that the primary product of the reaction is the  $\alpha$ -ketonic acid, which is subsequently reduced to the hydroxy-acid. Very recently the work was repeated in Cathcart's laboratory by Gertrude D. Bostock,<sup>3</sup> who in the main corroborated Lang's conclusions. This writer, however, makes a passing remark that the liver and intestinal mucosa failed to act on alanine.

Considerations which were discussed in a previous publication<sup>4</sup> led us to test the action of leucocytes and of various tissues on alanine under conditions in which bacterial growth was completely excluded. It was then definitely proven that under absolutely aseptic conditions, or under conditions of absolutely effective antiseptics, no deamination of alanine took place. Our observation was soon corroborated in Embden's laboratory.<sup>5</sup> This made it urgent to extend the experiments to a larger number of amino-acids. The experiments were carried out under aseptic conditions, and it was found that not one of the amino-acids tested suffered a deamination through the action of the leucocytes or of the kidney tissue.

<sup>1</sup> Lang: *Hofmeister's Beiträge*, v, p. 321, 1904.

<sup>2</sup> Neubauer: *Deutsch. Arch. f. klin. Med.*, xcv, p. 211, 1909.

<sup>3</sup> Gertrude D. Bostock: *Biochem. Journ.*, vi, p. 48, 1912.

<sup>4</sup> Levene and Meyer: *this Journal*, xv, p. 475, 1913.

<sup>5</sup> Griesbach and Oppenheimer: *Biochem. Zeitschr.*, lv. p. 329, 1913.

These observations make it necessary to repeat all the older work on deaminizing action of tissues. Experiments on purine bases are in progress.

#### EXPERIMENTAL.

*Tissues.* The leucocytes were obtained from dogs by the injection of turpentine into the pleural cavity. Rabbit kidneys were removed aseptically from exsanguinated animals. The tissues were finely minced before being added to the solutions.

*Solutions.* Glycocoll, aspartic acid, and leucine in approximately 2 per cent, and asparagine in 1 per cent concentration in 1 per cent Henderson phosphate mixture were used. Two kidneys were used in each experiment and control.

*Methods of analysis.* The amino-acid nitrogen was determined by the Van Slyke method, total nitrogen by the Kjeldahl process and ammonia nitrogen by distillation *in vacuo*. The details of analysis have been outlined in a previous communication.

*Bacteriological control.* Aerobic and anaerobic cultures and smears were made of all solutions. We wish to thank Dr. H. L. Amoss for his courtesy.

#### Kidney.

	ORIGINAL SOLUTION USED	VOLUME GAS	TEMPERATURE	PRESSURE	N	N GRAMS PER 100 cc.
	cc.	cc.	deg. C.	mm.	mgm.	
Phosphate solution:						
Before.....	10.0	2.40	22	759	1.35	0.013
After one week.....	10.0	2.60	20	750	1.46	0.015
Glycocoll solution:						
Before.....	2.5	17.10	22	759	9.64	0.385
After one week.....	2.5	17.20	20	750	9.65	0.386
Aspartic acid solution:						
Before.....	5.0	18.00	22	759	10.13	0.203
After one week.....	5.0	18.00	20	750	10.10	0.202
Asparagine solution:						
Before.....	5.0	16.20	20	758	9.30	0.184
After one week.....	5.0	16.20	21	760	9.18	0.184
Leucine solution:						
Before.....	5.0	18.00	20	758	10.24	0.205
After one week.....	5.0	18.30	21	760	10.35	0.207

*Leucocytes.*

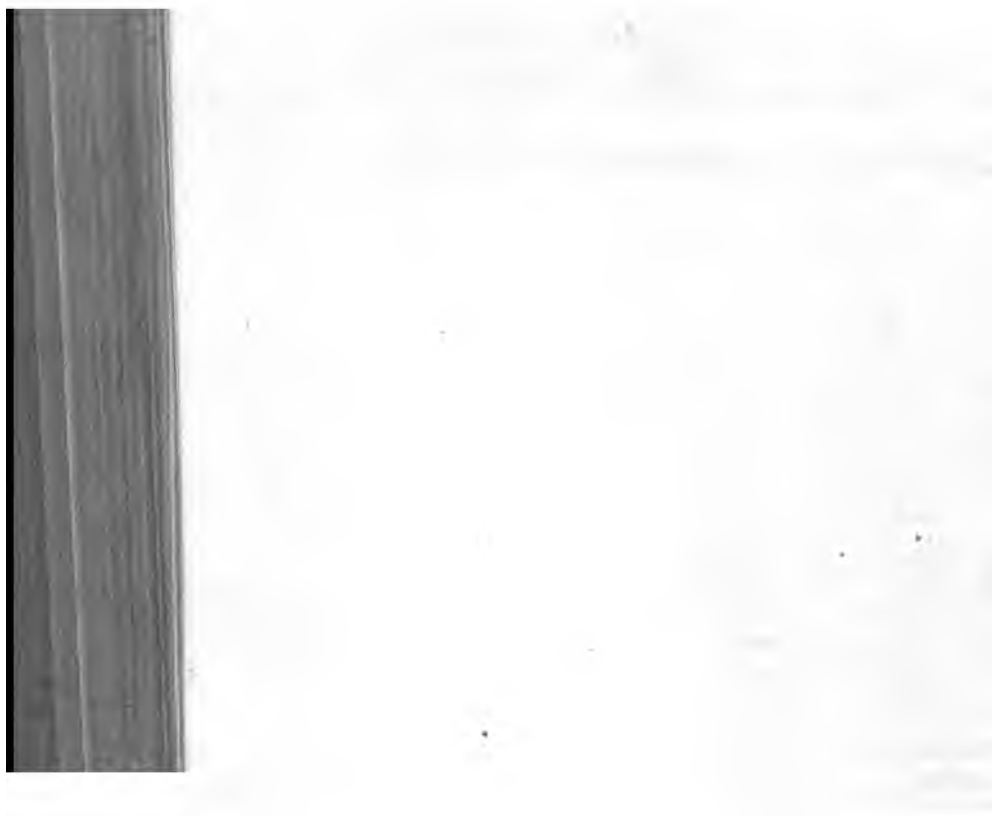
	ORIGINAL SOLUTION USED	VOLUME GAS	TEMPERATURE	PRESSURE	N	N GRAMS PER 100 CC.
<b>Leucocytes and phosphate solution:</b>						
Before.....	2.5	0.20	14	758	0.117	0.0468
After one week.....	2.5	0.20	12	752	0.117	0.0472
<b>Asparagine solution:</b>						
Before.....	2.5	8.50	14	758	4.980	0.1992
After one week.....	2.5	8.40	12	752	4.900	0.1960
<b>Leucine solution:</b>						
Before.....	2.5	11.40	14	758	6.680	0.2672
After one week.....	2.5	11.60	12	752	6.780	0.2712

*Kidney.*

SOLUTION	AMMONIA N PER CENT			AMINO N PER CENT			TOTAL N PER CENT		
	Before	After	Diff.	Before	After	Diff.	Before	After	Diff.
Phosphate..	0	0.005	0.005	0.013	0.015	0.002	0.025	0.038	0.013
Asparagine	0	0.006	0.006	0.184	0.184	0.000	0.112	0.122	0.010
Glycocoll..	0	0.008	0.008	0.385	0.386	0.001	0.186	0.198	0.012
Leucine.....	0	0.005	0.005	0.205	0.207	0.002	0.175	0.188	0.013
Aspartic acid.....	0	0.005	0.005	0.203	0.202	-0.001	0.366	0.378	0.012

*Leucocytes.*

Phosphate..	0	0.008	0.008	0.047	0.047	0.000	0.040	0.070	0.030
Asparagine	0	0.012	0.012	0.199	0.196	-0.003	0.168	0.201	0.033
Leucine.....	0	0.010	0.010	0.267	0.271	0.004	0.096	0.131	0.035





## ON "SUCRE VIRTUEL" AND BLOOD GLYCOLYSIS.

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In an interesting communication which recently appeared in this *Journal*, Macleod<sup>1</sup> states that according to Lépine and Barral "the concentration of actual sugar may be greater in blood that has stood for from fifteen minutes to an hour *at body temperature*,<sup>2</sup> outside the body than in freshly drawn blood," and he refers to page 64 of my book on diabetes (Paris, 1909). Neither on that page nor elsewhere in any of my publications can be found the words "at body temperature" but exactly in the middle of page 64 there appears the following title: AUGMENTATION DU GLYCOSE DANS LE SANG, *in Vitro*, à 58°. As a matter of fact, it is necessary to inhibit glycolysis and in order to obtain this result a temperature of 58° is essential; *but even this does not always prevent a great loss of sugar in the blood of certain dogs.*<sup>3</sup> But leaving aside for the moment exceptional cases, it may be said that if arterial blood be permitted to flow *simultaneously* by means of a bifurcated canula into two tared flasks, one of which, A, contains a known quantity of a solution of mercuric nitrate, while the other, B, immersed in water at 58–59°, contains a weighed quantity of water sufficient to prevent coagulation of the blood; and then if after a quarter, one-half, or even one hour the contents of flask B be poured into a solution of mercuric nitrate and the sugar determined in A and B, it is generally found that the amount of sugar per 1000 grams of blood is greater in B than in A. This

<sup>1</sup> J. J. R. Macleod: this *Journal*, xv, p. 497, 1913.

<sup>2</sup> Italics are mine.

<sup>3</sup> It is not easy to explain this exception. It is certain, as Barral and the writer showed in 1891, that the glycolytic enzyme nearly always loses its activity at 58°. It may be supposed that sometimes the enzyme which decomposes the actual sugar (see below) while continuing its diastatic action decomposes the sugar *which is present in a nascent state*. I advance this hypothesis with great reserve.

slight increase in sugar, commonly observed in healthy and normal dogs (in some instances, as mentioned above, a loss is observed), is much greater in dogs which have previously undergone an operation, particularly in such as have been bled.<sup>4</sup>

The increase in sugar is still greater if a few hours previous to the bleeding the animal is injected subcutaneously or intravenously with a small quantity of pancreatin, invertin, phlorhizin, adrenaline, morphine, or antipyrine—in short with any substance which brings about a rapid modification in the quantity of "sucre virtuel."

More interesting than the increase of sugar *in vitro* is that which occurs in the circulation. I discovered in 1903, in collaboration with Boulud, that in a dog that had been fasting about fifteen hours, the blood of the carotid (*i.e.*, of the left ventricle), contrary to the opinion of Cl. Bernard, very often contains more sugar than the blood of the right ventricle (obtained by means of a sound introduced into the right jugular vein). Many of our numerous experiments have made it possible to state that this increase of sugar in the blood of the carotid takes place at the expense of the combined sugar.<sup>5</sup> For example:

	SUGAR		
	Free	Combined	Total
Blood of the right ventricle .....	0.90	0.75	1.65
Blood of the carotid .....	1.10	0.50	1.60

The increase of sugar in the blood of the renal vein in phlorhizinized dogs discovered by Levene in 1895 (erroneously denied by Zuntz) has the same origin.<sup>6</sup> Recently we have demonstrated

<sup>4</sup> Macleod, having kept the blood at body temperature, naturally noted a loss of sugar which was due to normal glycolysis. In any case, if he had greatly increased the number of his experiments he might have found in some exceptional cases a slight gain. I have observed this in two or three instances out of more than one thousand experiments. This can be explained by assuming a considerable decomposition of "sucre virtuel" in an animal whose glycolytic power is weak.

<sup>5</sup> Regarding the difficulties in estimating the amount of the combined sugar, see Lépine and Boulud: *Journ. d. physiol. et d. path. gén.*, pp. 183-184, 1911.

<sup>6</sup> Lépine: *Revue d. méd.*, 1913, p. 614, *et seq.*; *Semaine méd.*, Sept. 24; Lépine and Boulud: *Compt. rend. de l'Acad. des Sci.*, October 6, 1913.

that the cleavage of the combined sugar is produced by an enzyme which can be extracted from the vascular wall.<sup>7</sup> This liberated sugar deserves the name of "sucre virtuel" inasmuch as it is ready to be utilized as soon as it is liberated (apparently spontaneously but in reality under the influence of an enzyme) from the combination in which it was present and in which combination it could not be detected by the ordinary reagents.

The enzyme concerned hydrolyzes phlorhizin. From this fact one is justified in drawing the conclusion that the "sucre virtuel" is of a glucosidic nature.

In reference to glycolysis, Macleod might well have added to his bibliography the article on *glycolysis* in the *Dictionnaire de physiologie* by Richet, and the chapter on *glycolysis* in my book on diabetes (pp. 152-191). According to Macleod the absence of glycolytic power of the serum was discovered by Rona and Döblin. This assertion is inaccurate because I carefully recorded this fact with Barral (in a note at the Académie des Sciences, 1890) and this is furthermore pointed out by Levene and Meyer (this *Journal*, xi, p. 364). With reference to the glycolytic activity of the leucocytes, which I was the first to discover, Van de Put might also be mentioned (*Arch. internat. d. physiol.*, ix, p. 292, 1910). Macleod studied glycolysis *in vitro* after having kept the blood for variable intervals at 40°. In any case, he should have made the estimations after one hour in order to have comparable results. He seems to deny the diminution of glycolysis in the blood of depancreatized dogs. I wish to refer him to page 357 of the *Journal de physiologie et de pathologie générale*, 1911. There he will find an explanation, at least a partial one, of the errors which may be committed in that respect (see also my book, p. 342).

I cannot finish without referring to the important fact discovered by Levene and Meyer that the sugar of the blood can be regained after glycolysis. I have observed an analogous fact (see *Journ. d. physiol. et d. path. gén.*, p. 184, 1911, note by Hugounenq and Morel). But this disturbing factor does not take place in the course of the first hour of glycolysis. Thus, no importance need be attached thereto, if the blood be left for only an hour in the incubator, as has always been my practice.

<sup>7</sup> Lépine and Boulud: *Compt. rend de l'Acad. des Sci.*, October 20, 1913.



## THE CHEMISTRY OF GLUCONEOGENESIS.

### VI. THE EFFECTS OF ACETALDEHYDE AND PROPYLALDEHYDE ON THE SUGAR FORMATION AND ACIDOSIS IN THE DIABETIC ORGANISM.<sup>1</sup>

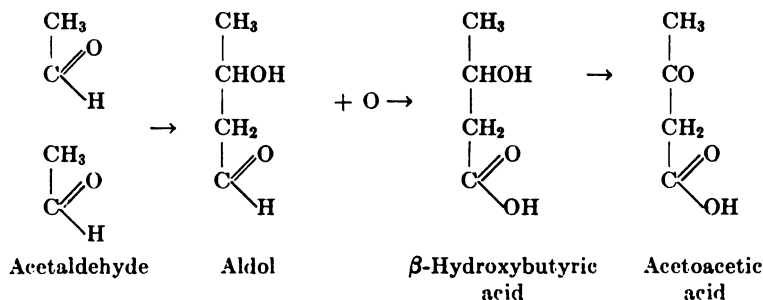
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(Received for publication, December 5, 1913.)

It has long been recognized that aldehydes are capable of effecting a great many syntheses in the animal and plant kingdoms. Thus, formaldehyde is generally accepted now to be the building stone from which sugars are synthesized in the plant kingdom<sup>2</sup> and Grube<sup>3</sup> demonstrated the possibility of this synthesis taking place in the liver of the turtle perfused with a fluid containing formaldehyde.

The first suggestion that acetaldehyde may play a rôle in the synthetic processes of the animal body was made by Spiro.<sup>4</sup> He suggested the possibility of  $\beta$ -hydroxybutyric acid arising from a condensation of two molecules of acetaldehyde, going through an aldol stage.



<sup>1</sup> Aided by a grant from the Rockefeller Institute for Medical Research.

<sup>2</sup> v. Bayer: *Ber. d. deutsch. chem. Gesellsch.*, iii, p. 63, 1870.

<sup>3</sup> Grube: *Pflüger's Archiv*, cxxi, p. 636, 1908; cxxxix, p. 428, 1911.

<sup>4</sup> Spiro, quoted by Magnus-Levy: *Arch. f. exp. Path. u. Pharm.*, xlii, p. 225, 1899.

Friedman<sup>5</sup> subjected these views to the test of experimentation and found that the perfusion of a dog's liver with blood to which acetaldehyde had been added, was actually followed by an increase in the acetoacetic acid in the perfusion mixture. On testing the effects of aldol he likewise obtained a very marked increase in the acetoacetic acid.

We were led to the study of the fate of acetaldehyde in the diabetic dog while searching for possible intermediary compounds in the metabolism of pyruvic acid. As was shown in a previous communication<sup>6</sup> pyruvic acid in different experiments does not yield glucose to the same degree. Several paths of catabolism of pyruvic acid suggested themselves, and we hoped to be able to show that pyruvic acid passes in part through acetaldehyde when it gives rise to only small quantities of glucose. The experiments, however, yielded entirely unexpected and contrary results.

The methods used in these experiments were the same as described in the previous communications of this series. Female dogs were used which were phlorhizinized by daily injection of 1 gram of phlorhizin ground up in olive oil. The animals were catheterized at the end of each period of twelve hours, after which the bladder was washed three or four times with warm distilled water. The acetaldehyde used was prepared by Merck (derivative of absolute alcohol) and was given subcutaneously diluted with water. In several instances the acetaldehyde was redistilled immediately before injection.<sup>7</sup> The nitrogen was determined by Kjeldahl, glucose by Alihn, ammonia by Folin, acetone, acetoacetic acid and  $\beta$ -hydroxybutyric acid by Shaffer's methods. The glucose determination was controlled by the polariscopic method, and after aldehyde feedings was also controlled for its fermentability by yeast.

#### *Effect of acetaldehyde, $\text{CH}_3\text{--CHO}$ .*

In experiment XXVII, period XIV, 8.8 grams ( $\frac{8}{5}$ ) of acetaldehyde dissolved in 35 cc. of water were given subcutaneously. From

<sup>5</sup> Friedman: *Hofmeister's Beiträge*, xi, p. 202, 1908.

<sup>6</sup> Ringer: *This Journal*, xv, p. 145, 1913.

<sup>7</sup> Attention must be called to the fact that acetaldehyde undergoes considerable deterioration on standing, which lessens its effects very materially.

the results of this experiment we see that the acetaldehyde exerts a very profound influence on the nitrogen as well as the glucose elimination. The nitrogen excretion of this dog which stayed above 5 grams per period for thirteen periods was reduced to 3.02 and 3.52 grams in periods XIV and XV to rise again to 6.60 grams in period XVI. The glucose elimination which was 18.75 grams in the foreperiod rose in the experimental period to 21.07 grams in spite of the very marked drop in the nitrogen excretion. The D : N ratio rose from its level of 3.65 in the foreperiod to 7.19 and 4.62 to come down again to 3.03 in period XVI. *The amount of extra glucose eliminated in periods XIV and XV was 16.10 grams.*

In experiment XXVIII, period IX, 8.8 grams ( $\frac{8}{5}$ ) of acetaldehyde dissolved in 25 cc. of water were given subcutaneously. Here as in the preceding experiment we note a depression in the nitrogen and a rise in the glucose elimination. The nitrogen elimination which was 6.42 grams in the foreperiod (period VIII) was reduced to 5.03 and 4.95 grams in period IX and X respectively to come up again to 6.18 and 6.23 in periods XI and XII. The glucose elimination which was 22.39 grams in the foreperiod, rose to 29.70 in spite of the reduction in the nitrogen elimination. The D : N ratio rose from 3.48 to 5.91 and 4.89 to come down again in periods XI and XII to 3.54 and 3.40. *The amount of extra glucose eliminated in periods IX and X was 18.9 grams.*

In this experiment the effect of acetaldehyde on the acidosis was also studied. As is seen from the table, page 576, acetaldehyde possesses a very marked antiketogenetic effect. The amount of  $\beta$ -hydroxybutyric acid elimination in the foreperiod was 2.35 grams. After the administration of acetaldehyde it was reduced to 1.42 and 0.50 gram in periods IX and X respectively, to come up again to 1.50 and 1.72 in periods XI and XII. Similar was the effect on the acetone and acetoacetic acid elimination. In period VIII (foreperiod) 630 mgm. of the two ketones were eliminated. After the acetaldehyde administration in periods IX and X it was reduced to 360 and 180 mgm. to rise again in periods XI and XII to 380 and 470 mgm. There was also a reduction in the ammonia elimination following the acetaldehyde administration. This was relative as well as absolute.

In experiment XXIX, period III, 8.8 grams ( $\frac{8}{5}$ ) of acetaldehyde dissolved in 28 cc. of water were given subcutaneously.

The results of this experiment corroborate our findings in the preceding two experiments. The nitrogen elimination which was 5.15 grams in the foreperiod (period II) was reduced to 3.27 and 2.77 in periods III and IV and rose again to 6.16 and 6.53 grams in periods V and VI. The glucose elimination, in spite of a very marked drop in the nitrogen output, rose from 17.59 in the foreperiod to 24.85 grams in period III resulting in a D : N ratio of 7.61. *The amount of extra glucose eliminated in periods III and IV was 20.45 grams.* The effect of the acetaldehyde on acidosis was also studied in this case. Although distinct, it was, however, not as marked as in experiment XXVIII. The reason for this probably lies in the fact that the elimination of acetone bodies in this dog was very low to start with.

In experiment XXX, period XII, 5.9 grams of acetaldehyde dissolved in 20 cc. of water were given subcutaneously. Here too the elimination of the acetone bodies and of nitrogen was depressed very considerably. The rise in the glucose output, however, was very slight, resulting in only 3.2 grams of extra glucose. It is noteworthy that this dog had a very low (for phlorhizin glucosuria), almost abnormal D : N ratio to start with, and we are inclined to believe that the failure to yield more extra glucose may have some relationship to it.

In experiment XXXI, period II, 8.8 grams ( $\frac{8}{5}$ ) of acetaldehyde dissolved in 30 cc. of water were given subcutaneously. There followed only a slight diminution in the nitrogen elimination, but a considerable rise in the glucose output. *The amount of extra glucose eliminated in periods II and III was 10.7 grams.*

On examining the tabulated results of these experiments we note a number of very striking effects brought about by the acetaldehyde.

I. A very marked depression of the nitrogen elimination which lasts for about two periods (twenty-four hours) after the acetaldehyde administration.

II. A rise in the absolute amount of glucose eliminated during the period of acetaldehyde administration, in spite of the drop in the nitrogen, accompanied by a very high rise in the D : N ratio.

III. A very marked depression in the acetone, acetoacetic acid and  $\beta$ -hydroxybutyric acid eliminations where acidosis is high.

As will be seen from a subsequent communication the admin-



istration to diabetic dogs of ethyl alcohol and acetic acid which stand in such very close chemical relationship to acetaldehyde are not followed by any of the effects enumerated above. This sug-

gested the possibility that the aldehyde radical  $-\text{C} \begin{array}{l} \text{O} \cdot \\ \diagup \\ \text{H} \end{array}$  may be responsible for the effects brought about by the acetaldehyde. We, therefore, decided to study the effects of its homologues.

*Effect of propylaldehyde,  $\text{CH}_3-\text{CH}_2-\text{CHO}$ .*

In experiment XXVII, period XVII, 11.6 grams ( $\frac{2}{3}$ ) of Kahlbaum's propylaldehyde dissolved in 30 cc. of water to which 3 cc. of ethyl alcohol had been added, were given subcutaneously. The results were very similar to those obtained with acetaldehyde. There followed a very marked depression in the nitrogen elimination and a rise in the D:N ratio. *The amount of extra glucose eliminated in periods XVII and XVIII was 11.65 grams.*

Much more convincing results were obtained in experiment XXVIII. In period XIII of this experiment 11.6 grams ( $\frac{2}{3}$ ) of propylaldehyde were administered as above. The nitrogen elimination which stood at the level of 6.18 and 6.23 in periods XI and XII dropped down to 3.27 and 4.83, to rise again to 6.24 grams in period XV. The glucose elimination in period XIII rose to 24.02 grams in spite of the very marked reduction in the nitrogen elimination, resulting in a rise in the D:N ratio to 7.35. *The amount of extra glucose eliminated was 19.09 grams.*

The effect of propylaldehyde on the ammonia, acetone, acetoacetic acid and  $\beta$ -hydroxybutyric acid eliminations was as marked as that of the acetaldehyde. All were depressed very considerably. The ammonia nitrogen elimination which was 0.65 and 0.70 gram in periods XI and XII was reduced to 0.25 gram in period XIII. The reduction of the ammonia nitrogen from 10.5 and 11.2 per cent to 7.6 per cent of the total nitrogen is also noteworthy. The acetone and acetoacetic acid elimination was reduced from 380 mgm. in period XI and 470 mgm. in period XII to 120 mgm. in periods XIII and XIV. The  $\beta$ -hydroxybutyric acid elimination which was 1.50 and 1.72 grams in the two foreperiods was reduced to 0.36 and 0.25 gram in periods XIII and XIV.

In experiment XXXII, period VI, 11.6 grams of propylaldehyde were administered as above. The results are very similar to those obtained in the preceding experiment. The glucose elimination and the D:N ratio rose very considerably, *yielding 19.75 grams of extra glucose*. The reduction in the acetone bodies was very marked. The acetone and acetoacetic acid having come down from 640 mgm. to a little above 200 mgm. while the  $\beta$ -hydroxybutyric acid was reduced from 1.87 grams in period V to 0.98 gram and 0.378 gram in periods VI and VII respectively.

*Effect of formaldehyde, H-CHO.*

We have attempted several experiments to study the effect of formaldehyde on the diabetic dog, but all have failed so far because of the toxicity of the aldehyde. The animal usually dies within twelve hours. We hope, however, to come back to these experiments in the near future by administering the aldehyde in very small quantities at a time. Perhaps this may yield satisfactory results.

*Discussion of results.*

From a review of the preceding pages we note a very remarkable reaction which apparently seems to be characteristic of substances possessing an aldehyde radical, for neither the alcohols, nor the acids corresponding to the aldehydes studied possess the power of effecting such deep seated changes in the metabolism of the diabetic animal. It is true that propyl alcohol and propionic acid possess the power of glucogenesis but they do not affect the nitrogen metabolism nor the acidosis, to the extent that propylaldehyde does. The effect of the acetaldehyde is much more remarkable, because neither the alcohol nor the acid that corresponds to it has any appreciable influence on the metabolism of the diabetic dog.

The outcome of our experiments leads to conclusions diametrically opposed to those of Friedman.<sup>8</sup> As was stated above he found that acetaldehyde on perfusion through the surviving liver gives rise to acetoacetic acid. On giving acetaldehyde subcutaneously, however, we found that it has just the opposite effect. How can we explain these differences?

<sup>8</sup> Friedman, *loc. cit.*

It is possible that in perfusion of the surviving extirpated liver one may deal with a metabolism that is decidedly abnormal, and which does not correspond with the process in the same organ when in normal condition. Under such circumstances, we can readily understand, why a substance should follow one path of metabolism in one case and an entirely different one in the other. It must also be borne in mind in this connection that the functions of the liver in the animal body may constantly be influenced by the other organs or by products of their metabolism. An illustration of such influence can be found in the works of Levene and Meyer<sup>9</sup> who showed how essential the coöperation of the different organs is for carbohydrate metabolism, and also to what erroneous and misleading conclusions one may be drawn by studying the influences of individual organs on the processes of metabolism, without taking into consideration the possible influence of the other organs upon the one studied.

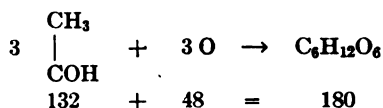
But most of the results obtained by Embden and his collaborators on perfused surviving livers have been found in a general way to be identical with those obtained in feeding experiments. Is it not possible, therefore, that in Friedman's experiments another factor played a rôle which is of great moment in determining the path of the metabolism of acetaldehyde? Is it not possible, for example, that the presence or absence of glycogen in the liver may have influenced his results? Surely, this is a matter which requires further study, especially since it was shown by Friedman<sup>10</sup> that the perfusion of sodium acetate through a liver poor in glycogen will cause an increase in the acetoacetic acid formation whereas the perfusion through a liver rich in glycogen will be followed by negative results.

*The glucogenetic effect of acetaldehyde and propylaldehyde.*

One of the most remarkable phenomena in our experiments is the very large amount of "extra" glucose that was eliminated after the administration of the acetaldehyde. If all the carbon of the acetaldehyde molecule were converted into glucose the administered 8.8 grams would give rise to 12 grams of glucose.

<sup>9</sup> Levene and Meyer: *This Journal*, ix, p. 97; xi, pp. 347, 353, 361; xii, p. 265.

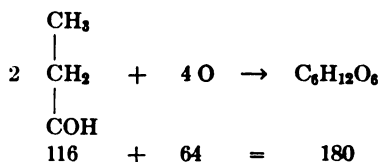
<sup>10</sup> Friedman: *Biochem. Zeitschr.*, lv, p. 436, 1913.



The amount of "extra" glucose found in experiment XXVII was 16.10 grams, in experiment XXVIII, 18.9 grams, in experiment XXIX, 20.45 grams and in experiment XXXI, 10.7 grams.

From this we see very clearly that considerably more glucose was eliminated in the urine after acetaldehyde administration, than can be accounted for by a complete conversion of the acetaldehyde into glucose. In other words, some substance or substances that are ordinarily non-gluco-genetic have contributed to the formation of glucose. The conclusion, therefore, seems justified that *acetaldehyde possesses the power of converting some substance in the animal metabolism that is non-gluco-genetic to one that is gluco-genetic and that the substance so formed possesses a greater number of carbon atoms than does acetaldehyde.* Whether acetaldehyde itself takes part in the gluco-genetic process will be discussed later.

What is true for acetaldehyde is also true for propylaldehyde, but to a lesser extent. If all the carbon of the propylaldehyde were converted into glucose 11.6 grams of propylaldehyde could yield 18 grams of glucose.



The amount of "extra" glucose obtained in experiment XXVII was 11.65 grams, in experiment XXVIII, 19.10 grams and in experiment XXXII, 19.75 grams. The "extra" glucose in experiment XXVII is lower than in any of the others and may be accounted for by the fact that it was performed on a dog that had had glucosuria for seventeen experimental periods, outside of the preparatory periods. At this time the animals find themselves in a very low state of vitality and this may account for the difference. The results obtained at this stage are usually taken for corroborative purposes only.

In experiments XXVIII and XXXII there is clearly a greater amount of glucose eliminated than can be accounted for by a complete conversion of the propylaldehyde into glucose, and similar to the acetaldehyde it seems to possess the power of converting non-glucogenetic substances into glucogenetic ones.

The question arises now, what is the nature of this change? In what way may the acetaldehyde or propylaldehyde exert its influence upon non-glucogenetic substances? Does the acetaldehyde or propylaldehyde bring about its effects by modifying the normal path of metabolism of those substances, or does it enter with them into a chemical union, thus changing their structural configuration and thereby modifying their path of catabolism?

When we come to examine the effects of the aldehydes on the diabetic organism as a whole and associate the different phenomena, a theory suggests itself which seems to harmonize all the facts. Our experiments bring to light three important facts:

I. That the administration of aldehydes in diabetic animals is followed by a very marked rise in the glucose elimination.

II. That concomitant with this phenomenon there is a considerable drop in the elimination of acetone bodies.

III. That alcohols and acids related to the aldehydes do not possess these effects.

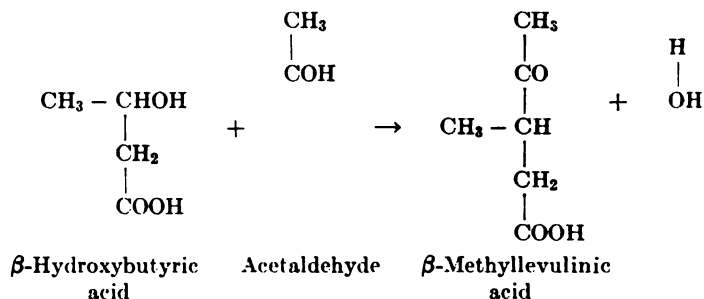
From the above it becomes evident that the aldehyde radical

$-\text{C} \begin{array}{l} \nearrow \text{O} \\ \searrow \text{H} \end{array}$  is the determining factor in bringing about the effects

described. In a subsequent communication additional evidence will be presented in support of this view. The aldehyde radicals possess great combining powers and it is generally recognized now, in what a complexity and multiplicity of unions the aldehydes are capable of entering. In the disaccharides, glucosides, and glucuronates and a great many other compounds this fact is evident.

When we bear this fact in mind and realize that the acetaldehyde and propylaldehyde in our diabetic animals have brought about a diminution in the acetone bodies on the one hand and an increase of the glucose elimination on the other, it seems reasonable to assume that the two phenomena may be causally related,

*i.e.*, the aldehydes, because of their great combining power, may have the property of combining with the secondary alcohol radical of  $\beta$ -hydroxybutyric acid, and by changing its structural configuration convert it into a substance that is glucogenetic. An illustration of the possibility is given in the following reaction,



which results in the formation of  $\beta$ -methyllevulinic acid, *i.e.*, the conversion of a normal fatty acid into an iso compound. It has been shown by Baer and Blum, Embden and his collaborators, and by Ringer, Frankel and Jonas<sup>11</sup> that the iso compounds in the animal body undergo demethylation.  $\beta$ -Methyllevulinic acid would therefore be converted into levulinic acid, which, as will be shown in a subsequent communication, does possess glucogenetic properties.

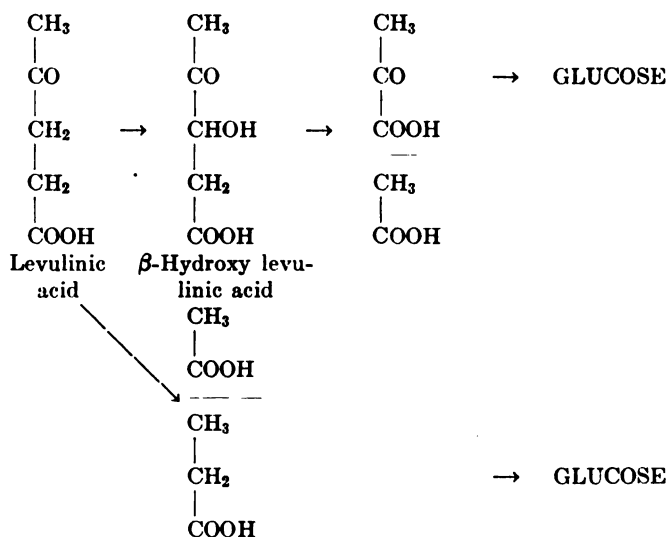
It must be realized that between the  $\beta$ -hydroxybutyric acid and acetaldehyde combination and levulinic acid there may be a number of possible intermediary compounds of the keto and enol forms which will be discussed elsewhere. At present we wish only to sketch our conception of the possible reaction and indicate the possibility of the conversion of a compound with an even number of carbon atoms as  $\beta$ -hydroxybutyric acid to one with an uneven number of carbon atoms, the conversion of an acetone-genetic compound to one that is glucogenetic.

Objection may be raised to this theory because the increase of glucose elimination in our experiment is much greater than is the

<sup>11</sup> Baer and Blum: *Arch. f. exp. Path. u. Pharm.*, lv, p. 89, 1906; Embden, Salomon and Schmidt: *Hofmeister's Beiträge*, viii, p. 129; Ringer, Frankel and Jonas: *This Journal*, xiv, p. 525, 1913.

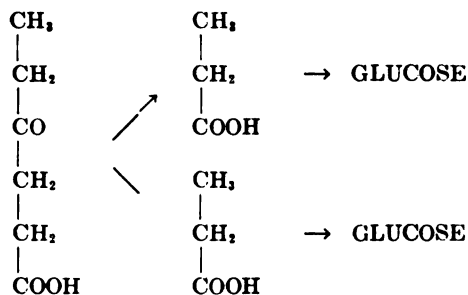
drop in the acetone elimination. But as will be shown in a subsequent communication, this objection is not valid. For, the acetone bodies that are eliminated in the urine are only a small fraction of that which actually plays a part in the intermediary metabolism, and any change that is evident in the urine may be greatly magnified in the intermediary metabolism.

The path of levulinic acid in metabolism is the subject of our present inquiry. It may be one of the following; it may undergo  $\beta$ -oxidation giving rise to pyruvic acid, or it may undergo further



oxidation in the  $\gamma$ -carbon, breaking up into acetic acid and propionic acid. In either case we get a three-carbon compound which is glucogenetic. Judging from the amount of glucose we obtain after acetaldehyde administration we are inclined to believe that propionic acid is the final product.

In the case of the propylaldehyde we may conceive of the following reactions:



These experiments have opened up a new field for experimentation which, in our estimation, throws a great deal of light on the mechanism of antiketogenesis. They also bring up the question of the influence of the higher aldehydes on metabolism. Most of these experiments are completed and will be published in the near future. The effect of the aldehydes on the protein metabolism will be discussed in a separate communication.

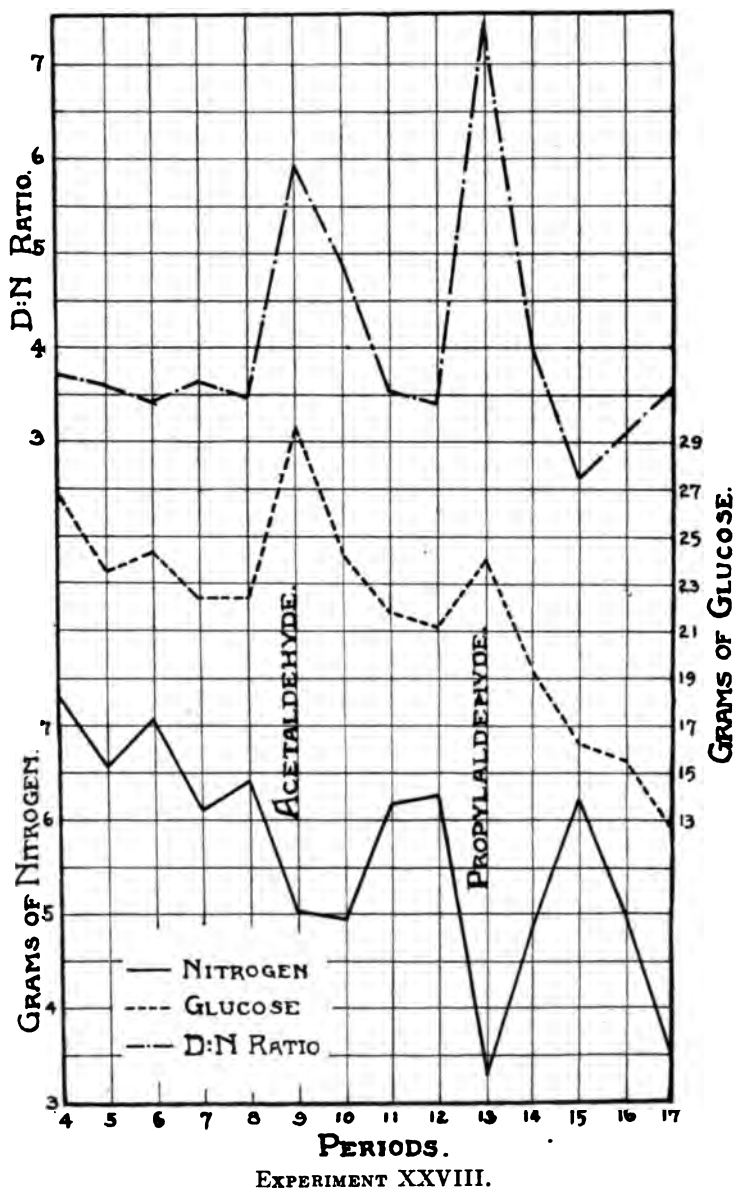


EXPERIMENT XXVII. *Twelve-hour periods.*

DATE MAY, 1913	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL GLUCOSE	D. N.	EXTRA GLUCOSE	REMARKS
18	VIII		6.15	24.26	3.95		
18	IX	11.35	6.42	23.95	3.73		
19	X		6.15	21.30	3.46		
19	XI	10.90	5.82				
20	XII		5.26	17.55	3.34		
20	XIII	10.64	5.14	18.75	3.65		
21	XIV		3.02	21.70	7.19	16.10	8.8 grams acetaldehyde dissolved in 30 cc. of water given subcutaneously.
21	XV		3.52	16.24	4.62		
22	XVI		6.00	19.87	3.03		
22	XVII		3.14	15.64	4.98	11.65	11.6 grams propylaldehyde dissolved in 30 cc. of water to which 3 cc. of ethyl alcohol had been added, given subcutaneously.
23	XVIII		3.78	15.04	3.98		
23	XIX		3.12	7.74	2.48		

## EXPERIMENT XXVIII. Twelve-hour periods.

DATE MAY, 1913	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL GLUCOSE	D: N	EXTRA GLUCOSE	AMMONIA NITROGEN	NH <sub>4</sub> N: TOTAL N IN %	ACETONE AND ACETO- ACETIC ACID	$\beta$ -HYDROXY BUTYRIC ACID	REMARKS
18	IV	12.13	7.33	26.96	3.68		0.49	6.7	0.30	1.75	
19	V		6.56	23.50	3.58		0.44	6.7	0.32	1.86	
19	VI	11.48	7.07	24.38	3.45		0.40	5.7	0.40		
20	VII		6.11	22.36	3.66		0.61	10.0	0.76		
20	VIII	11.00	6.42	22.39	3.48		0.73	11.3	0.63	2.35	
21	IX		5.03	29.70	5.91		0.43	8.5	0.36	1.42	8.8 grams acet- aldehyde dis- solved in 25 cc. of water given subcutaneous- ly.
21	X	10.53	4.95	24.20	4.89	18.9	0.41	8.3	0.18	0.50	
22	XI		6.18	21.88	3.54		0.65	10.5	0.38	1.50	
22	XII	10.45	6.23	21.14	3.40		0.70	11.2	0.47	1.72	
23	XIII		3.27	24.02	7.35		0.25	7.6	0.12	0.36	11.6 grams of pro- pyl aldehyde dissolved in water and alco- hol as above, given subcu- taneously.
23	XIV		4.83	19.45	4.03	19.09			0.12	0.25	
24	XV		6.24	16.27	2.62				0.15	0.35	
24	XVI	9.77	4.99	15.50	3.11				0.23	0.51	
25	XVII		3.55	12.76	3.60						



EXPERIMENT XXIX. Twelve-hour periods.

DATE JUNE, 1913	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL GLUCOSE	D: N	EXTRA GLUCOSE	ACETONE AND ACETO- ACETIC ACID	$\beta$ -HYDROXY BUTYRIC ACID	REMARKS
3	I						0.070	0.143	
3	II	11.00	5.15	17.59	3.42		0.078	0.200	
4	III		<b>3.27</b>	<b>24.85</b>	<b>7.61</b>	20.45	<b>0.062</b>	<b>0.170</b>	8.8 grams acetaldehyde dissolved in .28 cc. of water given subcuta- neously.
4	IV		<b>2.77</b>	<b>13.90</b>	<b>5.02</b>		<b>0.043</b>	<b>0.143</b>	
5	V		6.16	16.24	2.64		0.038	0.090	
5	VI	10.68	6.53	18.43	2.82		0.079	0.184	

EXPERIMENT XXX. Twelve-hour periods.

DATE MAY, 1913	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL GLUCOSE	D: N	EXTRA GLUCOSE	ACETONE AND ACETO- ACETIC ACID	$\beta$ -HYDROXY BUTYRIC ACID	REMARKS
9	VII	8.55	3.59	8.98	2.50		0.33	0.79	
10	VIII		3.53	8.56	2.44		0.47	1.34	
10	IX	8.36	2.73						
11	X		2.62	7.20	2.75				
11	XI	8.34	2.79	7.60	2.72		0.36	1.13	
12	XII		<b>1.97</b>	<b>7.83</b>	<b>3.97</b>	3.2	<b>0.14</b>	<b>0.38</b>	5.0 grams acetaldehyde dissolved in 20 cc. of water given subcu- taneously.
12	XIII		<b>1.77</b>	<b>5.57</b>	<b>3.14</b>		<b>0.06</b>	<b>0.11</b>	

## EXPERIMENT XXXI. Twelve-hour periods.

DATE MAY, 1913	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL GLUCOSE	D: N	EXTRA GLUCOSE	ACETONE AND ACETO- ACETIC ACID	$\beta$ -HYDROXY BUTYRIC ACID	REMARKS
14	I		5.97	22.50	3.77		0.06	0.18	8.8 grams acetaldehyde dissolved in 30 cc. of water given subcu- taneously.
15	II		5.49	26.35	4.80	10.7	0.10	0.39	
15	III	12.76	5.76	26.25	4.56		0.07	0.18	
16	IV		7.80	28.60	3.68		0.13	0.35	

## EXPERIMENT XXXII. Twelve-hour periods.

DATE OCTOBER, 1913	PERIOD	WEIGHT	TOTAL NITROGEN	TOTAL GLUCOSE	D: N	EXTRA GLUCOSE	ACETONE AND ACETO- ACETIC ACID	$\beta$ -HYDROXY BUTYRIC ACID	POLARI- SATION	REMARKS
17	IV	15.24	7.25	28.65	3.96		0.735	2.73	+1.06°	11.6 grams propylaldehyde dissolved in water and alcohol as above given subcutaneously.
17	V		7.90	29.37	3.72		0.640	1.87	+1.19°	
18	VI	14.96	7.04	38.25	5.44	19.75	0.288	0.98	+1.53°	
18	VII		6.32	32.40	5.12		0.211	0.38	+1.33°	
19	VIII	15.02	8.70	33.96	3.90		0.216	0.38	+1.42°	
19	IX		8.75	29.19	3.34		0.202	0.31	+1.24°	



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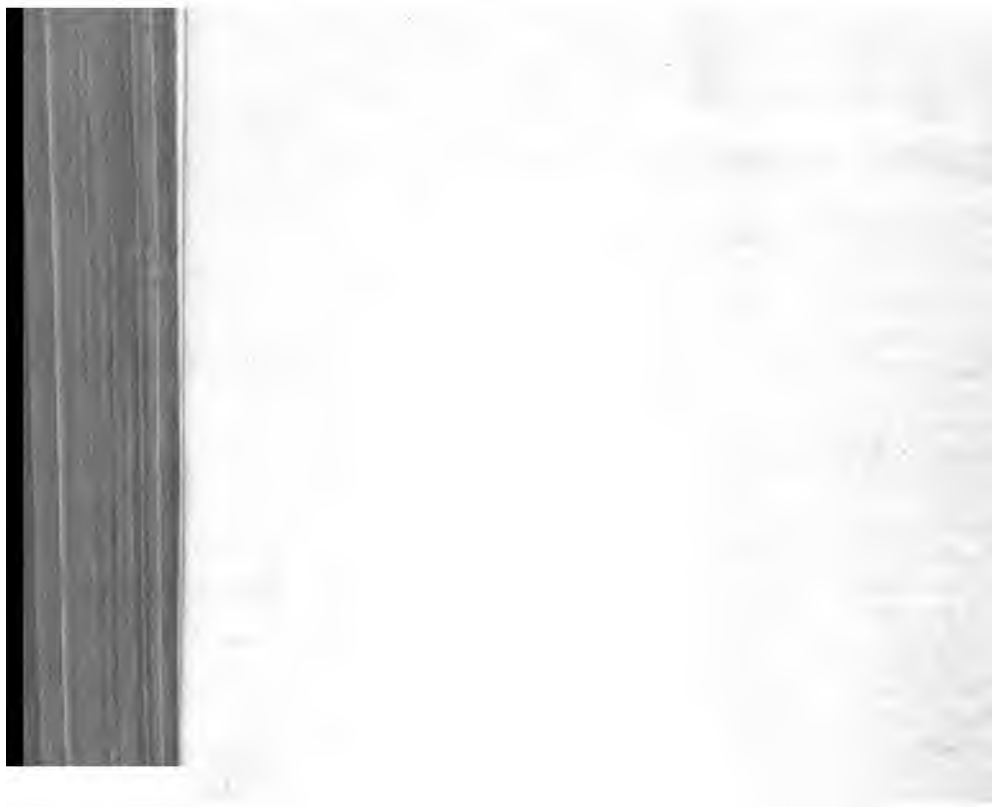
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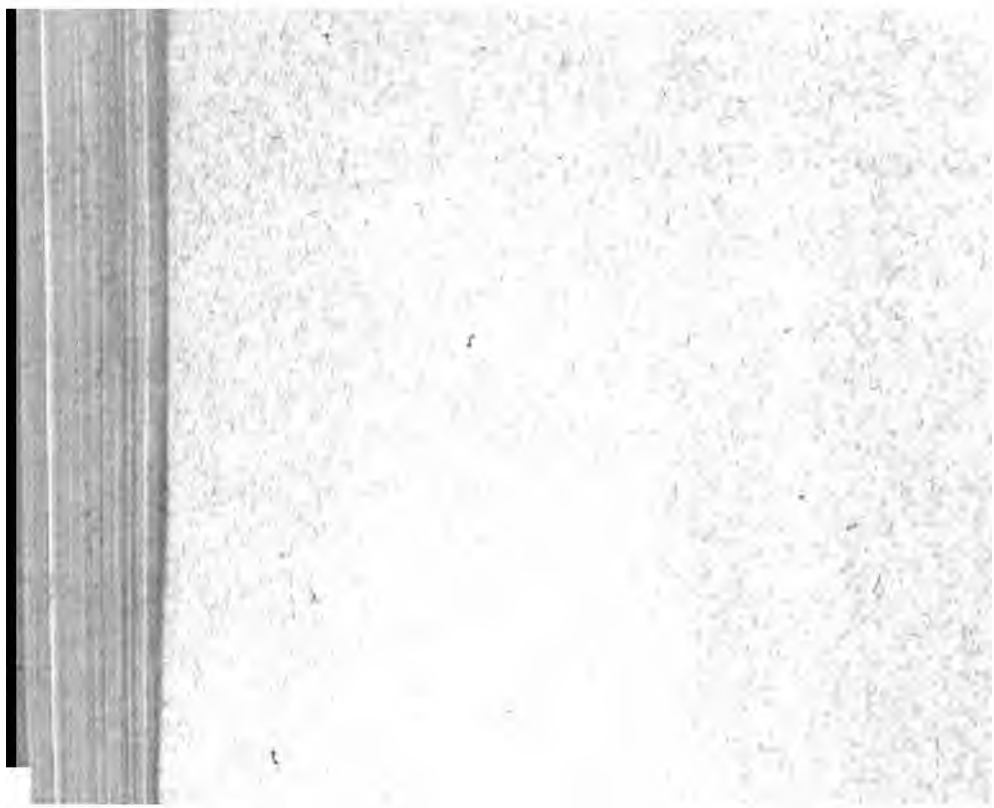
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